

**Investigation of factors influencing the
successful persistence and dissemination
of a globally distributed antibiotic
resistance plasmid**

By

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**A thesis submitted to the University of Birmingham for the
degree of DOCTOR OF PHILOSOPHY**

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September 2011

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Abstract

In Gram negative bacteria, resistance to β -lactam antibiotics is predominantly mediated by β -lactamases. CTX-M-14 is one of the most frequently identified extended spectrum β -lactamases (ESBLs) worldwide and is produced by *Enterobacteriaceae* isolated from humans and animals. In 2004, an IncK plasmid (named pCT) carrying *bla*_{CTX-M-14} was isolated from cattle in the UK. This plasmid was shown to successfully persist and spread within diverse *E. coli* strains from livestock and the environment on a dairy farm. The aim of this study was to determine the extent of pCT dissemination, and to investigate the factors which allow pCT to stably persist within bacterial populations.

Complete DNA sequencing and annotation revealed that plasmid pCT was 93,629 bp in size and encoded all three IncI group associated regions, the *repYZ*, *tra* and *pil* loci. No genes known to play a role in determining virulence were identified, and sequencing revealed *bla*_{CTX-M-14} as the only antimicrobial drug resistance gene on pCT. Comparison of the pCT genome to other *bla*_{CTX-M} encoding plasmids showed no conserved regions with the exception of the antibiotic resistance gene itself. From this information, a PCR assay for rapid and high-throughput detection of pCT was designed, and applied to a collection of isolates from several countries. These assays showed that pCT has disseminated worldwide in bacteria from humans, animals and the environment.

pCT was stably maintained in the absence of β -lactam antibiotic pressure in four different bacterial host strains over ~70 generations, and conferred neither a fitness advantage nor disadvantage to two host *E. coli* strains. Within a third *E. coli* host strain however, pCT did confer a fitness cost, although this was shown to be in part due to the presence of the antibiotic resistance gene *bla*_{CTX-M-14}. The addition of pCT also had no significant effect on any bacterial host phenotype investigated, other than susceptibility β -lactams.

Seven pCT genes of interest in relation to plasmid success were identified, including the antibiotic resistance gene *bla*_{CTX-M-14}, conjugation related genes, a putative sigma factor and a putative toxin-antitoxin system *pndACB*. These genes were inactivated using an adapted recombination method designed during this study to investigate their role in the 'success' of this plasmid. Inactivation of conjugation pilus genes *traXY* prevented successful plasmid transfer in all conditions tested; however, the inactivation of the second pilus loci only reduced the frequency of conjugation in liquid to some of the recipient strains. Inactivation of the five other pCT genes had no consistent effect on the host bacterial strain persistence or dissemination.

In conclusion, pCT was shown to be a globally successful plasmid, distributed in *E. coli* isolated from humans, animals and the environment. The persistence and spread of this plasmid is postulated to be due to a combination of subtle factors such as stability within a range of hosts, a lack of a fitness burden conferred to new host strains, and the proficient conjugation of pCT in liquid and on solid media, rather than any one particular gene or phenotypic benefit conferred to the host.

For Lucy,

Whose strength, determination and optimism
are an inspiration to me every single day!

Acknowledgements

I am extremely grateful to my supervisors, Laura Piddock, who gave me this opportunity, supported and directed me, and who has taught me more than I will probably ever comprehend, and to Mark Webber, whose wise words, unending guidance, support and sense of humour were simply invaluable.

I would like to thank members of ARG old and new, in particular Vito and Bailey who listened to me wittering on and helped wherever they could; to Leanne for being my β -lactamase buddy, and Jess for her sage advice and constant help, both day-to-day and in a crisis.

Also to Donna, Cyn, Raga, Alistair, Alan M, Vikki, Alison and Alan Tranter for being AMAZING!

Thanks also to Martin Woodward and Nick Coldham at the VLA for their useful input and constant support; to Chris Thomas from Biosciences for his time spent teaching me the infamous 'caesium chloride' technique; to Ian Henderson for his support, wise and kind words; to Theresa Morris for help with the SEM; to Elizabeth Marsh for teaching me the ways of the worms, and to Dafydd Taylor for being a great student and helping me out immensely.

I would also like to thank my family for their continuous support as I shuffle around the country/globe, especially my mum who is always on the end of the phone at one in the morning. My BFFs Lucy, Fee and Flick, and all my PhD buddies Ella, Lucy, Sarah, Chris, Will and Greg for forcing me to leave the lab and go to the junction every once in a while. Also to Claire and Andrea (*B. cereus* about rice safety!) who make me glad to be a geek, and Kevin and Nic who always support me all the way from Canada!

And finally,

a huge thanks to Tim Wells for always being there for a cup of tea whenever I needed him, but for also giving me the confidence to think everything might be possible,

to Grace, who's support, friendship and sense of fun has sustained me throughout my project and who's proof reading skills are second to none,

and to Amelia, who makes me laugh every day and has allowed me to keep some of my sanity throughout this process. Without Amelia this PhD would have been utterly impossible!

Clarification of Contribution to Collaborative work

This study was conducted as a collaboration with the Animal Health and Veterinary Laboratories agency (AHVLA). Meetings every three months with Dr Nick Coldham and Prof. Martin Woodward provided guidance and advice throughout the project.

Sequencing of the pCT genome was also part of a collaboration with the Sanger Institute (Cambridge). Purified plasmid DNA was sent to the Sanger Institute by JLC. 454 sequencing, assembly and initial annotation was conducted by Anna M. Cerdeño-Tárraga, Heidi Hauser and Nicholas R. Thomson at the Sanger Institute. Subsequent annotation and analysis in this thesis were by JLC.

Under the supervision of JLC, undergraduate student Dafydd Taylor (University of Birmingham) conducted PCR assays to optimise the multiplex pCT-detection method, first using DNA from plasmid pCT and then screening the UK veterinary isolates and Spanish clinical isolates for pCT-like plasmids. All figures in the present thesis relating to this work were generated by JLC.

Cells for visualisation using Scanning electron microscopy were prepared by JLC, then dehydrated and mounted using platinum fixing by Theresa Morris at the School of Metallurgy and Materials, University of Birmingham. Use of the Scanning electron microscope and images generated for this thesis were by JLC.

Table of Contents

| | | |
|-----------|---|----|
| 1. | Introduction | |
| 1.1 | <i>Enterobacteriaceae</i> | 1 |
| 1.1.1 | <i>E. coli</i> | 2 |
| 1.2 | Antibiotics | 3 |
| 1.2.1 | β -lactams | 4 |
| 1.2.2 | Mechanisms of resistance to antibiotics | 7 |
| 1.2.2.1 | Mobilisation of antibiotic resistance genes | 8 |
| 1.3 | β -lactamases | 8 |
| 1.3.1 | Classification of β -lactamases | 10 |
| 1.3.2 | Extended spectrum β -lactamases (ESBLs) – Ambler class A/ Group 2be | 12 |
| 1.3.3 | CTX-M β -lactamases | 13 |
| 1.3.3.1 | Activity and structure of CTX-M β -lactamases | 14 |
| 1.3.3.2 | Origins of <i>bla</i> _{CTX-M} | 15 |
| 1.3.3.3 | Epidemiology of <i>bla</i> _{CTX-M} | 17 |
| 1.3.3.4 | Clonal expansion of strains carrying <i>bla</i> _{CTX-M} | 19 |
| 1.3.3.5 | Acquisition, carriage and reservoirs of CTX-M producing bacteria | 20 |
| 1.3.3.6 | CTX-M producing bacteria from animals | 22 |
| 1.3.4 | CTX-M-14 | 23 |
| 1.3.4.1 | Emergence and epidemiology | 24 |
| 1.3.4.2 | <i>bla</i> _{CTX-M-14} mobilisation | 25 |
| 1.4 | Plasmids | 26 |
| 1.4.1 | Characterisation of plasmids | 26 |
| 1.4.1.1 | Incompatibility and <i>rep</i> typing | 27 |
| 1.4.1.2 | MOB relaxase typing | 28 |
| 1.4.1.3 | Plasmid multi-locus sequence typing (pMLST) | 28 |
| 1.4.2 | Incompatibility group I complex plasmids | 29 |
| 1.4.2.1 | Complete DNA sequences of IncI complex plasmids | 29 |
| 1.4.3 | Epidemiology of plasmids carrying antibiotic resistance genes | 30 |
| 1.4.4 | Plasmid evolution and evolutionary fitness | 31 |
| 1.4.5 | Plasmid control measures | 34 |
| 1.4.6 | Plasmid mediated <i>bla</i> _{CTX-M-14} | 36 |
| 1.4.6.1 | <i>bla</i> _{CTX-M-14} on IncK plasmids | 37 |
| 1.5 | Background to this study, <i>E. coli</i> isolate C159/11 and <i>bla</i> _{CTX-M-14} carrying IncK plasmid pCT | 38 |
| 1.6 | Hypotheses | 39 |
| 1.7 | Aims | 40 |

| | | |
|-----------|--|----|
| 2. | Materials and Methods | |
| 2.1 | Bacterial strains and growth conditions | 41 |
| 2.2 | Determination of susceptibility to antibiotics | 46 |
| 2.3 | Plasmid DNA isolation | 48 |
| 2.3.1 | Mini plasmid preparation | 48 |
| 2.3.2 | Maxi plasmid preparation | 48 |
| 2.3.3 | Isolation of high quality plasmid DNA for sequencing | 48 |
| 2.3.3.1 | Birnboim and Doly maxi-preparation | 48 |
| 2.3.3.2 | Caesium chloride density gradient centrifugation | 49 |
| 2.4 | Complete plasmid sequence analysis | 50 |
| 2.4.1 | Sequencing and annotation | 50 |
| 2.4.2 | Comparative genomics | 51 |
| 2.5 | Amplification and DNA sequencing of genomic elements on pCT | 51 |
| 2.5.1 | Polymerase chain reaction | 51 |
| 2.5.2 | Agarose gel electrophoresis | 52 |
| 2.5.3 | Sequencing of amplimers | 55 |
| 2.5.4 | PCR assays to identify pCT-like plasmids | 55 |
| 2.5.5 | Relaxase (<i>nikB</i>) typing and phylogenetic analysis | 56 |
| 2.6 | Transfer of pCT into new host strains | 56 |
| 2.6.1 | Transformation of pCT into <i>E. coli</i> DH5 α | 56 |
| 2.6.2 | Selection of rifampicin resistant mutants | 58 |
| 2.6.3 | Conjugation of pCT into <i>E. coli</i> J53-2, <i>E. coli</i> 3950 and <i>S. Typhimurium</i> SL1344 <i>rif</i> ^R | 59 |
| 2.7 | Further characterisation of pCT-like plasmids | 60 |
| 2.8 | Inactivation of genes on pCT | 61 |
| 2.8.1 | Design and generation of the DNA integrative cassette with the antibiotic resistance selective marker | 61 |
| 2.8.2 | Design and generation of the DNA integrative cassette encoding <i>gfp</i> | 63 |
| 2.8.3 | Recombineering and selection of mutant plasmids | 63 |
| 2.8.4 | Confirmation of gene inactivation | 65 |
| 2.8.4.1 | Detection of β -lactamase production | 65 |
| 2.8.5 | Transfer of pCT mutant plasmids pCT2-6 and pCT8 to bacterial host strains <i>E. coli</i> J53-2, <i>E. coli</i> 3950 and <i>S. Typhimurium</i> SL1344 <i>rif</i> ^R | 66 |
| 2.9 | Characterisation of plasmid stability and transfer | 67 |
| 2.9.1 | Plasmid stability <i>in vitro</i> | 67 |
| 2.9.2 | Plasmid conjugation frequencies | 67 |
| 2.10 | Effect of pCT and mutant pCT upon host phenotype | 69 |
| 2.10.1 | Bacterial host growth rate | 69 |
| 2.10.2 | Biofilm formation assays | 70 |
| 2.10.2.1 | Crystal violet staining | 70 |
| 2.10.2.2 | Culture on agar containing Congo red | 71 |
| 2.10.3 | Settle assay to determine the aggregative ability of strains | 71 |
| 2.10.4 | Imaging of cells | 71 |

| | | |
|-----------|--|-----|
| 2.10.5 | Effect of pCT and the pCT2-9 upon the ability of bacterial host strains to cause infection | 72 |
| 2.10.5.1 | Tissue culture model | 72 |
| 2.10.5.2 | <i>C. elegans</i> model | 76 |
| 2.11 | Pair-wise competitive growth <i>in vitro</i> | 78 |
| 3. | Characterisation and epidemiology of plasmid pCT | |
| 3.1 | Background | 80 |
| 3.2 | Hypotheses and Aims | 80 |
| 3.3 | Preparation of bacterial strains containing pCT | 81 |
| 3.3.1 | Transformation of <i>E. coli</i> DH5 α with pCT | 81 |
| 3.3.1.1 | Conjugation of pCT into <i>E. coli</i> and <i>S. Typhimurium</i> strains | 84 |
| 3.4 | Antibiotic susceptibilities of pCT containing host strains | 85 |
| 3.5 | Genomic analysis of plasmid pCT | 85 |
| 3.5.1 | Isolation of pCT plasmid DNA for sequencing | 85 |
| 3.5.2 | Complete DNA sequencing and annotation | 88 |
| 3.5.3 | General features of pCT | 88 |
| 3.5.4 | Antibiotic resistance and virulence genes | 95 |
| 3.5.4.1 | The genetic context of <i>bla</i> _{CTX-M-14} on pCT | 95 |
| 3.5.5 | Replication genes | 97 |
| 3.5.6 | Stability and persistence genes | 98 |
| 3.5.6.1 | <i>impA/B/C</i> UV protection or mutation repair genes | 98 |
| 3.5.7 | Transfer and conjugation genes | 98 |
| 3.5.7.1 | <i>tra</i> operon | 99 |
| 3.5.7.2 | <i>pil</i> operon | 99 |
| 3.5.8 | Other pCT regions | 101 |
| 3.5.8.1 | Putative sigma factor | 101 |
| 3.5.8.2 | Other insertion sequences | 101 |
| 3.5.9 | Comparisons with other plasmids within the IncI complex | 103 |
| 3.5.10 | Comparisons of pCT with other <i>bla</i> _{CTX-M} encoding plasmids | 109 |
| 3.6 | New diagnostic tool to detect plasmid pCT-like plasmids | 116 |
| 3.6.1 | Design of a specific PCR assay for detection of the pCT backbone | 116 |
| 3.6.2 | <i>bla</i> _{CTX-M-14} and flanking regions | 117 |
| 3.6.3 | Validation of the PCR assay to detect pCT-like plasmids | 118 |
| 3.7 | pCT global epidemiology | 122 |
| 3.7.1 | Phylogenetic analysis using <i>nikB</i> sequences | 122 |
| 3.7.2 | Further characterisation of the pCT-like plasmids | 124 |
| 3.8 | Discussion | 124 |
| 3.9 | Further work | 137 |
| 3.10 | Key findings | 139 |

| | | |
|-----------|---|-----|
| 4. | Factors affecting plasmid pCT persistence, and fitness of its bacterial hosts | |
| 4.1 | Background | 140 |
| 4.2 | Hypotheses and Aims | 140 |
| 4.3 | The effect of the addition of pCT on bacterial host phenotype | 141 |
| 4.3.1 | Growth of bacterial host strains +/- pCT | 141 |
| 4.3.2 | Co-culture of bacterial host strains +/- pCT | 141 |
| 4.3.3 | <i>In vitro</i> competition between <i>E. coli</i> DH5α and DH5α pCT (non-transferrable) | 144 |
| 4.3.4 | The ability of bacterial host strains (+/- pCT) to cause infection | 144 |
| 4.4 | Plasmid pCT persistence and transfer | 149 |
| 4.4.1 | Persistence of pCT in bacterial host cells | 149 |
| 4.4.2 | pCT transfer rates | 149 |
| 4.4.2.1 | Optimisation of pCT conjugation methods | 149 |
| 4.4.2.2 | Frequency of pCT conjugative transfer to various recipient bacterial strains | 151 |
| 4.5 | pCT conjugation rates in the presence and absence of cefotaxime | 154 |
| 4.6 | Discussion | 154 |
| 4.7 | Further work | 164 |
| 4.8 | Key findings | 168 |
| 5. | The contribution of <i>bla</i>_{CTX-M-14} to the biology of pCT | |
| 5.1 | Background | 169 |
| 5.2 | Hypotheses and Aims | 170 |
| 5.3 | Development of a method to inactivate pCT <i>bla</i> _{CTX-M-14} | 171 |
| 5.4 | Inactivation of pCT <i>bla</i> _{CTX-M-14} by insertion of <i>aph</i> | 172 |
| 5.5 | Inactivation of pCT <i>bla</i> _{CTX-M-14} with a <i>gfp</i> | 176 |
| 5.6 | Removal of <i>bla</i> _{CTX-M-14} upstream insertion sequence <i>ISEcp1</i> | 179 |
| 5.7 | Effects on pCT plasmid biology of inactivating <i>bla</i> _{CTX-M-14} and <i>ISEcp1</i> | 180 |
| 5.7.1 | Stability of plasmids pCT2, pCT2b and pCT6 in host cells | 180 |
| 5.7.2 | Conjugative transfer of pCT2, pCT2b and pCT6 compared to pCT | 180 |
| 5.8 | Effects of pCT2, pCT2b and pCT6 upon the bacterial host strain compared to wild-type pCT | 183 |
| 5.8.1 | Susceptibility to cefotaxime and ceftiofur | 183 |
| 5.8.2 | Growth rate of host bacteria containing pCT2, pCT2b and pCT6 | 183 |
| 5.8.3 | The effect of pCT2, pCT2b and pCT6 on the ability of bacterial host strains to cause infection | 189 |
| 5.9 | Pair-wise competition <i>in vitro</i> of hosts containing pCT and pCT2 | 192 |
| 5.10 | <i>In vitro</i> competition between <i>E. coli</i> J53-2 and <i>E. coli</i> J53-2 pCT2 | 192 |
| 5.11 | Discussion | 195 |
| 5.12 | Further work | 204 |
| 5.13 | Key findings | 207 |

| | | |
|-----------|---|------------|
| 6. | The role of five pCT genes in the “success” of pCT | |
| 6.1 | Hypotheses and Aims | 209 |
| 6.2 | Inactivation of five pCT ‘backbone’ genes | 209 |
| 6.2.1 | Selection of genomic regions for further investigation | 209 |
| 6.2.1.1 | Putative sigma factor | 209 |
| 6.2.1.2 | The <i>pil</i> locus and <i>pilS</i> | 210 |
| 6.2.1.3 | Shufflon region and shufflon recombinase (<i>rci</i>) | 210 |
| 6.2.1.4 | The <i>tra</i> locus and <i>traY</i> | 211 |
| 6.2.1.5 | Putative addiction system <i>pndACB</i> | 211 |
| 6.2.2 | Inactivation of five pCT genes and verification | 212 |
| 6.2.3 | Creation of a mutant with both <i>traXY</i> and <i>pndACB</i> inactivated | 214 |
| 6.3 | Effects on pCT plasmid biology of inactivating the five genes | 214 |
| 6.3.1 | Stability of pCT3, pCT4, pCT5, pCT7, pCT8 and pCT9 in bacterial cells | 214 |
| 6.3.2 | Conjugative transfer of pCT3, pCT4, pCT5, pCT7, pCT8 and pCT9 | 215 |
| 6.4 | Effects of pCT3, pCT4, pCT5, pCT7, pCT8 and pCT9 upon bacterial host strains | 217 |
| 6.4.1 | Susceptibility of strains containing mutant plasmids to cefotaxime and ceftiofur | 217 |
| 6.4.2 | Growth kinetics of host strains containing pCT3-5 and pCT7-9 | 217 |
| 6.4.3 | The effect of pCT3, pCT4, pCT5 and pCT8 on the ability of bacterial host strains to cause infection | 221 |
| 6.5 | Competition of pCT3- 9 against wild-type pCT <i>in vitro</i> | 224 |
| 6.6 | Pair-wise competition between <i>E. coli</i> DH5α and DH5α pCT9 <i>in vitro</i> | 224 |
| 6.7 | The role of pCT and the <i>pil</i> and <i>tra</i> locus in host biofilm formation and aggregation | 227 |
| 6.7.1 | The ability of host strains containing pCT and pCT2-7 to form a biofilm | 227 |
| 6.7.1.1 | Crystal violet staining | 227 |
| 6.7.1.2 | The production of curli and cellulose using Congo red staining | 228 |
| 6.7.2 | Aggregation of bacterial hosts containing plasmids pCT and pCT2-6 | 231 |
| 6.7.3 | Visualisation of cells using Scanning Electron Microscopy (SEM) | 236 |
| 6.8 | Discussion | 240 |
| 6.9 | Further work | 252 |
| 6.10 | Key findings | 254 |
| 7. | Overall Discussion | 256 |
| | Publications resulting from this study | 266 |
| | Appendix | 267 |
| | References | 304 |

List of Tables

Chapter 1 Introduction

| | | |
|-----------|-----------------------------------|----|
| Table 1.1 | β -lactamase classification | 11 |
|-----------|-----------------------------------|----|

Chapter 2 Materials and Methods

| | | |
|-----------|--|----|
| Table 2.1 | Bacterial host strains +/- wild-type pCT | 42 |
| Table 2.2 | Other bacteria strains used in this study | 43 |
| Table 2.3 | <i>E. coli</i> isolates investigated for the presence of pCT-like plasmids | 44 |
| Table 2.4 | Bacterial strains containing mutant pCT plasmids (pCT2-9) | 45 |
| Table 2.5 | Antimicrobials used in this study | 47 |
| Table 2.6 | Primers used for amplification of specific regions on pCT and detection of pCT-like plasmids | 53 |
| Table 2.7 | Primers used in construction of mutant plasmids and conformation of recombination | 54 |

Chapter 3 Characterisation and epidemiology of plasmid pCT

| | | |
|-----------|--|-----|
| Table 3.1 | Minimum Inhibitory Concentrations of a range of antibiotics to strains with and without plasmid pCT | 86 |
| Table 3.2 | Protein coding regions of pCT listed clockwise | 90 |
| Table 3.3 | pCT complete sequence homology across whole genomes of available sequenced plasmids | 104 |
| Table 3.4 | Summary of PCR assays for the detection of pCT-like plasmids | 121 |
| Table 3.5 | Further characterisation of pCT-like plasmids, the ability to conjugate and transferable resistance profiles | 125 |

Chapter 5 The contribution of *bla*_{CTX-M-14} to the biology of pCT

| | | |
|-----------|---|-----|
| Table 5.1 | Minimum inhibitory concentrations of two β -lactam antibiotics for hosts containing pCT and <i>bla</i> _{CTX-M-14} modified plasmids pCT2, pCT2b and pCT6 | 184 |
|-----------|---|-----|

Chapter 6 The role of five pCT genes in the 'success' of pCT

| | | |
|-----------|--|-----|
| Table 6.1 | Minimum inhibitory concentrations of cefotaxime and ceftiofur for hosts containing pCT and the other modified plasmids | 218 |
| Table 6.2 | The proportion of cells with protrusions visualised using scanning electron microscopy | 238 |

List of Figures

| | | |
|------------------|---|-----|
| Chapter 1 | Introduction | |
| Figure 1.1 | Common antibiotic classes used to treat humans and animals | 5 |
| Figure 1.2 | The structure of β -lactam antibiotics and action of β -lactamase | 6 |
| Figure 1.3 | Ribbon diagram of CTX-M-9 | 16 |
| Figure 1.4 | Global distribution of <i>bla</i> _{CTX-M} variants the and relative proportions within each region | 18 |
| Chapter 2 | Materials and Methods | |
| Figure 2.1 | Primers used to determine the site of <i>bla</i> _{CTX-M-14} insertion on pCT-like plasmids | 57 |
| Figure 2.2 | Inactivation of selected pCT genes by homologous recombination with a PCR amplified DNA product. | 62 |
| Figure 2.3 | Typical pair-wise competitive growth method | 79 |
| Chapter 3 | Characterisation and epidemiology of plasmid pCT | |
| Figure 3.1 | Plasmid DNA isolated from <i>E. coli</i> C159/11 and the transformed <i>E. coli</i> DH5 α pCT | 82 |
| Figure 3.2 | <i>bla</i> _{CTX-M} Group-9 PCR detection in putative pCT containing strains | 83 |
| Figure 3.3 | Isolation of pCT plasmid DNA | 87 |
| Figure 3.4 | Plasmid map of pCT | 89 |
| Figure 3.5 | GC % of <i>bla</i> _{CTX-M-14} gene and surrounding regions | 96 |
| Figure 3.6 | CDS organisation of the pCT transfer genes (<i>tra</i> , <i>trb</i> and <i>pil</i>) | 100 |
| Figure 3.7 | DNA sequence comparison of pCT vs. R721 | 102 |
| Figure 3.8 | DNA sequence comparison of pCT vs. pO26_vir | 105 |
| Figure 3.9 | DNA sequence comparison of pCT vs. pR3521 | 106 |
| Figure 3.10 | DNA sequence comparison of pCT vs. pO113 | 107 |
| Figure 3.11 | DNA sequence comparison of pCT vs. R64 | 110 |
| Figure 3.12 | DNA sequence comparison of pCT vs. R387 | 111 |
| Figure 3.13 | DNA sequence comparison of pCT vs. TP113 | 112 |
| Figure 3.14 | DNA sequence comparisons of pCT vs. other <i>bla</i> _{CTX-M} carrying plasmids | 114 |
| Figure 3.15 | DNA sequence comparison of pCT vs. pEK204 | 115 |

| | | |
|------------------|--|-----|
| Figure 3.16 | PCR detection of pCT-like plasmids in veterinary isolates | 119 |
| Figure 3.17 | Amplification of the <i>bla</i> _{CTX-M-14} insertion region <i>traK</i> to <i>ISEcp1</i> and <i>bla</i> _{CTX-M-14} to the adjacent pseudogene | 120 |
| Figure 3.18 | Relaxase phylogenetic typing | 123 |
| Figure 3.19 | Comparison of pCT (IncK) and pR3521 (IncB) antisense loop sequence and structure | 132 |
| Figure 3.20 | Summary map of the epidemiology of pCT-like plasmid identified from <i>E. coli</i> isolates | 135 |
| Chapter 4 | Factors affecting plasmid pCT persistence, and fitness of its bacterial host | |
| Figure 4.1 | Growth kinetics of host strains +/- pCT | 142 |
| Figure 4.2 | The proportion of pCT carrying strains within a population | 143 |
| Figure 4.3 | Pair-wise competitive growth <i>in vitro</i> between <i>E. coli</i> DH5 α and DH5 α containing pCT7 (non-conjugative pCT) | 145 |
| Figure 4.4 | The ability of <i>S. Typhimurium</i> SL1344, SL1344 <i>rif</i> ^R and SL1344 <i>rif</i> ^R containing pCT to adhere to and invade human intestinal cells | 147 |
| Figure 4.5 | The ability of bacterial hosts +/- pCT to cause infection in <i>C. elegans</i> | 148 |
| Figure 4.6 | pCT conjugation frequency time optimisation on solid media | 150 |
| Figure 4.7 | Conjugation frequencies of pCT from donor <i>E. coli</i> DH5 α to various recipients on solid and in liquid media | 152 |
| Figure 4.8 | Conjugation frequencies of pCT from donor <i>E. coli</i> C159/11 to various recipients on solid and in liquid media | 153 |
| Figure 4.9 | Conjugation frequencies on solid media of pCT in the presence of sub-inhibitory concentrations of cefotaxime | 155 |
| Figure 4.10 | Conjugation frequencies in liquid media of pCT in the presence of sub-inhibitory concentration of cefotaxime | 156 |
| Chapter 5 | The contribution of <i>bla</i>_{CTX-M-14} to the biology of pCT | |
| Figure 5.1 | PCR amplimers from SW102 colonies containing putative mutated pCT (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 173 |
| Figure 5.2 | DNA sequence showing the successful insertion of the <i>aph</i> gene into <i>bla</i> _{CTX-M-14} on pCT | 175 |
| Figure 5.3 | Nitrocefin test for β -lactamase production | 177 |
| Figure 5.4 | PCR amplimers from <i>E. coli</i> SW102 colonies containing putative mutated pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) separated by electrophoresis | 178 |

| | | |
|------------------|---|-----|
| Figure 5.5. | Conjugation frequencies of pCT and <i>bla</i> _{CTX-M-14} mutants pCT2, pCT2b and pCT6 on solid media to various recipients | 181 |
| Figure 5.6 | Conjugation frequencies of pCT and <i>bla</i> _{CTX-M-14} mutants pCT2, pCT2b and pCT6 in liquid media to various recipients | 182 |
| Figure 5.7 | Growth kinetics of <i>E. coli</i> 3950 containing plasmids pCT2, pCT2b and pCT6 compared to <i>E. coli</i> 3950 pCT | 185 |
| Figure 5.8 | Growth kinetics of <i>E. coli</i> J53-2 containing plasmids pCT2, pCT2b and pCT6 compared to <i>E. coli</i> J53-2 pCT | 186 |
| Figure 5.9 | Growth kinetics of <i>E. coli</i> DH5α containing plasmids pCT2, pCT2b and pCT6 compared to <i>E. coli</i> DH5α pCT | 187 |
| Figure 5.10 | Growth kinetics of <i>S. Typhimurium</i> rif ^R containing plasmids pCT2, pCT2b and pCT6 compared to <i>S. Typhimurium</i> rif ^R SL1344 pCT | 189 |
| Figure 5.11 | The ability of <i>S. Typhimurium</i> SL1344 rif ^R with wild-type pCT and <i>bla</i> _{CTX-M-14} modified plasmids to adhere to and invade human intestinal cells | 190 |
| Figure 5.12 | The ability of bacterial hosts containing pCT2, pCT2b and pCT6 to cause infection the <i>C. elegans</i> | 191 |
| Figure 5.13 | Competition between <i>E. coli</i> hosts carrying pCT and pCT2 | 193 |
| Figure 5.14 | Pair-wise competitive growth <i>in vitro</i> between <i>E. coli</i> J53-2 and <i>E. coli</i> J53-2 pCT2 (<i>bla</i> _{CTX-M-14::aph}) | 194 |
| Figure 5.15 | Sequence of the pCT ISE _{cp1} element and downstream spacer region marked with putative promoters and the site for recombination | 203 |
| Chapter 6 | The role of five pCT genes in the ‘success’ of pCT | |
| Figure 6.1 | pCT genes selected for inactivation | 213 |
| Figure 6.2 | Conjugation frequencies of plasmids from donor <i>E. coli</i> DH5α to recipients <i>E. coli</i> J53-2, <i>E. coli</i> 3950 and <i>S. Typhimurium</i> SL1344 rif ^R | 216 |
| Figure 6.3 | Growth kinetics of <i>E. coli</i> DH5α containing pCT and pCT3-9 | 219 |
| Figure 6.4 | Growth kinetics of host strains containing pCT, pCT3, pCT4 and pCT5 | 220 |
| Figure 6.5 | The ability of <i>S. Typhimurium</i> SL1344 rif ^R containing wild-type pCT, pCT3, pCT4, pCT5 and pCT8 to adhere to and invade human intestinal cells | 222 |
| Figure 6.6 | The ability of bacterial hosts containing pCT3, pCT4, pCT5 and pCT8 to cause infection in <i>C. elegans</i> | 223 |

| | | |
|-------------|---|-----|
| Figure 6.7 | Competition Index of plasmids pCT3-9 when competed <i>in vitro</i> against wild-type pCT | 225 |
| Figure 6.8 | Pair-wise competitive growth <i>in vitro</i> between <i>E. coli</i> DH5α and <i>E. coli</i> DH5α pCT9 (<i>tra::aph</i> ; <i>pndAC::cat</i>) | 226 |
| Figure 6.9 | Biofilm formation of <i>E. coli</i> C159/11 and <i>E. coli</i> 3950 containing the plasmids pCT and pCT2-6 | 229 |
| Figure 6.10 | Biofilm formation of <i>E. coli</i> DH5α containing the plasmids pCT and pCT2-7. | 230 |
| Figure 6.11 | Colony morphology of bacterial hosts strains containing plasmid pCT and plasmid mutants (pCT2-7) grown on LB agar containing Congo red | 232 |
| Figure 6.12 | The aggregative ability of <i>S. Typhimurium</i> SL1344 rif ^R and <i>E. coli</i> J53-2 +/- pCT | 233 |
| Figure 6.13 | The aggregative ability of <i>S. Typhimurium</i> SL1344 rif ^R and <i>E. coli</i> J53-2 +/- pCT4 and pCT5 | 234 |
| Figure 6.14 | Aggregative ability of <i>S. Typhimurium</i> SL1344 rif ^R and <i>E. coli</i> J53-2 +/- pCT2, pCT2b, pCT3 and pCT6 | 235 |
| Figure 6.15 | Scanning electron microscopy images of DH5α +/- pCT from LB broth | 237 |
| Figure 6.16 | Scanning electron microscopy images of SL1344 rif ^R +/- pCT from LB broth | 239 |
| Figure 6.17 | Scanning electron microscopy images of <i>E. coli</i> DH5α pCT and <i>E. coli</i> DH5α pCT7 cells from LB agar | 241 |
| Figure 6.18 | Scanning electron microscopy images of C159/11 from broth and agar | 242 |
| Figure 6.19 | The DNA sequence of the pCT of shufflon region | 247 |

List of Abbreviations

| Abbreviation | Definition |
|---------------------------------|--|
| aa | Amino acid |
| ACC | Ambler class C |
| amp | Ampicillin |
| <i>aph</i> | Aminoglycoside phosphotransferase |
| API | Analytical profile index |
| bp | Base pair |
| BSAC | British Society for Antimicrobial Chemotherapy |
| <i>cat</i> | Chloramphenicol acetyl transferase |
| Cat | Catalogue |
| CFU | Colony forming units |
| CI | Competition index |
| CMY | Cephamecinases |
| CsCl | Caesium Chloride |
| CTX | Cefotaxime |
| CTX-M | Cefotaximase |
| DHA | Dhahran hospital Saudi Arabia |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| EAEC | Enterocaggregative <i>E. coli</i> |
| EDTA | Ethylenediaminetetraacetic acid |
| EPEC | Enteropathogenic <i>E. coli</i> |
| ESBL | Extended spectrum β -lactamase |
| EtBr | Ethidium Bromide |
| FOX | Cefoxitinase |
| g | Gram |
| <i>g</i> | Gravity |
| GES | Guiana extended spectrum |
| GFP | Green Fluorescent Protein |
| H ₂ S | Hydrogen sulphide |
| HBSS | Hanks balanced salt solution |
| HCl | Hydrochloric acid |
| IMI | Imipenem hydrolysing |
| IMP | Imipenemase |
| Inc | Incompatibility |
| INT-407 | Human intestinal epithelial cell line |
| ISO | Iso-sensitest |
| kan | Kanamycin |
| kb | Kilobase |
| KDa | Kilodalton |
| KH ₂ PO ₄ | Monopotassium phosphate |
| KPA | <i>Klebsiella pneumoniae</i> group A |
| KPC | <i>K. pneumoniae</i> carbapenemase |
| kV | Kilovolts |
| L | Litre |
| LB | Luria Bertani |
| M | Molar |
| MDa | Mega Daltons |
| MDR | Multi-Drug resistant |

| Abbreviation | Definition |
|----------------------------------|---|
| MEM | Minimum Essential Medium |
| mg | Milligram |
| MOX | Moxalactamase |
| MIC | Minimum Inhibitory Concentration |
| min | Minute |
| ml | Millilitre |
| mM | Millimolar |
| mRNA | Messenger Ribonucleic acid |
| Na ₂ HPO ₄ | Disodium hydrogen phosphate |
| NaCl | Sodium chloride |
| NDM | New-Delhi metallo |
| NCTC | National collection of typed cultures |
| NEAA | Non essential amino acids |
| ng | Nanogram |
| NMI | Not metalloenzyme carbapenemase |
| no | Number |
| OD | Optical density |
| OXA | Oxacillinase |
| <i>P</i> | Probability |
| PBP | Penicillin binding protein |
| PBS | Phosphate buffered saline |
| PER | P. Nordmann, E. Ronco R. Labia |
| PSE | <i>Pseudomonas</i> -specific enzymes |
| PCR | Polymerase chain reaction |
| pl | Isoelectric point |
| rif | Rifampicin |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RT-PCR | Reverse transcriptase PCR |
| SD | Standard deviation |
| SDW | Sterile distilled water |
| sec | Second |
| SHV | Sulfhydryl variant |
| SME | <i>Serratia marcescens</i> enzyme |
| TBE | Tris-Borate EDTA buffer |
| TE | Tris EDTA buffer |
| TEM | Temoniera |
| TNE | Tris (NaCl) EDTA buffer |
| Tris | <i>Tris</i> (hydroxymethyl)aminomethane |
| UV | Ultra Violet |
| V | Voltage |
| VIM | Verona integron encoded metallo |
| XLD | Xylose Lysine Deoxycholate agar |
| Ω | Ohm (measure of resistance) |
| μF | Microfarad (measure of capacitance) |
| μg | Microgram |
| μl | Microlitre |
| μm | Micrometer |
| °C | Degrees centigrade |

Chapter 1:

Introduction

1. Introduction

1.1 *Enterobacteriaceae*

The *Enterobacteriaceae* are a group of Gram-negative, rod shaped bacteria within the Gamma Proteobacteria. *Enterobacteriaceae* members are all facultative aerobes, are non-sporulating, are either non motile or motile by peritrichous flagella, and include the genera *Escherichia*, *Salmonella*, *Klebsiella*, *Proteus*, *Yersinia* and *Enterobacter*. While the *Enterobacteriaceae* are a relatively homogeneous group they are able to occupy a range of different ecological niches and cause a variety of different human pathologies. Many *Enterobacteriaceae* species are ubiquitous in the environment and can be identified from soils, water and decaying vegetation. Members such as *Escherichia coli*, *Proteus spp.*, *Enterobacter spp.* or *Klebsiella spp.* are found as part of the normal intestinal flora of healthy humans and animals (Costa *et al.*, 2008). However, species regarded as commensals can infrequently cause opportunistic disease in their hosts, most commonly in the immuno-compromised or when the bacteria are found outside the gastrointestinal tract (Ronald, 2002; Kaper *et al.*, 2004). Certain *Salmonella enterica* serovars are commensal bacteria in poultry and reptiles, but are pathogenic to humans, causing gastroenteritis (Hohmann, 2001). Others are host restricted and cause severe pathology in humans, such as *Salmonella enterica* serovar Typhi and also *Shigella spp.*, which clusters indistinguishably with *E. coli* sub-species during phylogenetic analysis (Crump *et al.*, 2004; von Seidlein *et al.*, 2006; Lan and Reeves, 2002). An important feature of the *Enterobacteriaceae*, relevant to this study, is the ability of mobile genetic elements to transfer between them. This allows the sharing and dissemination of an extensive accessory genome between diverse bacterial species and across a range of

environmental niches that they occupy (Jones and Sneath, 1970; Sanderson, 1976).

1.1.1 *E. coli*

E. coli is perhaps the *Enterobacteriaceae* species most intrinsically associated with humans. Neonates are typically colonised with *E. coli* strains within hours of birth, and a diverse range of strains remain in the human colon as part of the healthy flora (Gill *et al.*, 2006; Palmer *et al.*, 2007). However, there are several *E. coli* subtypes or 'pathotypes' which harbour virulence genes encoding, for example, the production of toxins, colonisation factors and adhesins which increase the likelihood that the *E. coli* clone will cause disease in humans (Rasko *et al.*, 2008). Three clinical syndromes are commonly associated with infection by particular pathotypes, urinary tract infections, systemic infections including meningitis and sepsis, and diarrhoeal disease (Kaper *et al.*, 2004). While the number of *E. coli* urinary tract infections caused every year are unknown due to underreporting and difficulties in diagnosis, UTIs are considered the most prevalent bacterial infections, and *E. coli* the most common cause, therefore the disease burden is thought to be extensive (Foxman, 2002). *E. coli* is also a significant cause of bacteraemia and was identified as the causative agent in 27,055 cases in the UK in 2010, accounting for ~30% of all bacteraemia cases that year (HPA, 2011). Among the diarrhoeagenic *E. coli* there are at least six well described categories; enteroaggregative *E. coli* (EAEC), enteropathogenic *E. coli* (EPEC), enteroinvasive *E. coli* (EIEC), enterohemorrhagic *E. coli* (EHEC), enterotoxigenic *E. coli* (ETEC) and *Shigella* dysentery which have distinct clinical presentations. ETEC is an important cause of diarrhoea in the developing world, particularly in children under five where infection by this bacteria causes millions of cases of gastroenteritis and tens of thousands of deaths each year

(Black, 1993). ETEC is also thought to account for approximately a third of 'traveller's diarrhoea' cases in travellers from developed countries (Shah *et al.*, 2009). EHEC, due to a low infective dose, severe disease presentation and resultant sequelae, is also a relevant pathogen in the developed world where these strains have been responsible for numerous outbreaks (Pennington, 2010); and Shigellosis causes over 125 million cases of dysentery annually, 5-15% of which result in a fatality (Barhan *et al.*, 2010). Many of these pathotypes are able to reside as commensals in the gastrointestinal tract of animals. As *E. coli* are also a major constituent of the normal flora and excreta of livestock (Sorum and Sunde, 2001), these strains can be found in the farm environment, and may become part of the food chain within contaminated water, food from animal origin or fresh food products such as leafy salads (O'Brien *et al.*, 2001; Erickson and Doyle, 2007; Berger *et al.*, 2010). Many *E. coli* infections do not require treatment with antibiotics as they may be self limiting therefore requiring simple fluid replacement, or in the case of ETEC strain antibiotics may aggravate the symptoms of the disease (Guerrant *et al.*, 2001; Porter *et al.*, 2011). However persistent or systemic infections require antibiotic treatment in order to resolve the bacterial infection (BNF60, 2011).

1.2 Antibiotics

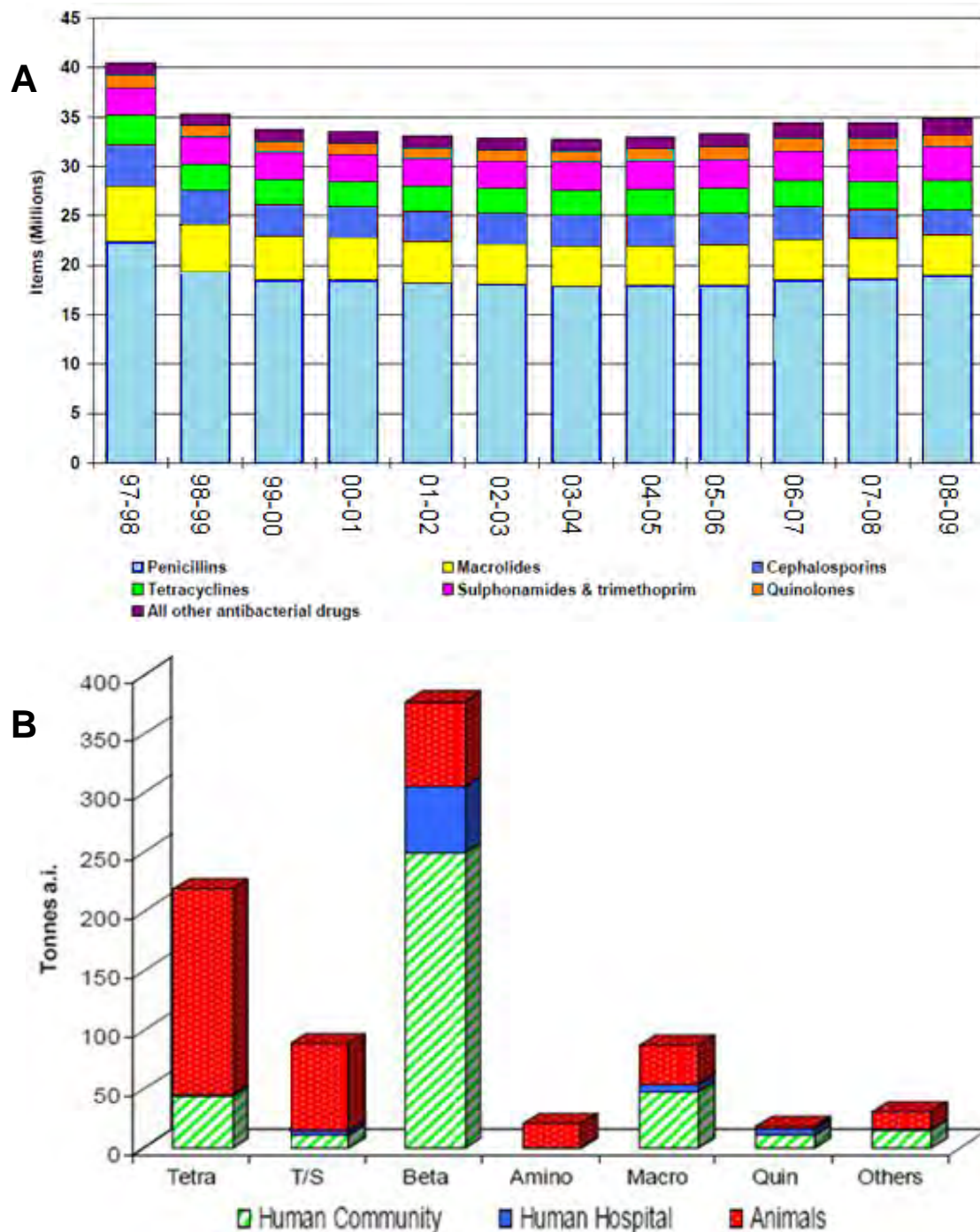
Antibiotics are defined as substances which selectively destroy or inhibit the growth of microorganisms. This description encompasses an array of molecules with diverse modes of action including the prevention of bacterial protein synthesis (e.g. aminoglycosides, tetracyclines, macrolides and chloramphenicol); inhibition of nucleic acid synthesis (sulphonamides, quinolones, rifamycins); disrupted cell membrane function (polymyxins) and inhibition of bacterial cell wall synthesis (β -lactams and

glycopeptides). Drugs belonging to each antibiotic class contribute to both human and veterinary treatment regimes. In UK clinical medicine β -lactams are the most frequently prescribed antibiotic (Figure 1.1), comprising 66% of community treatments, 77% of antibiotics used in hospital and equating to ~198 tonnes of active ingredient per year (DEFRA, 2007; NHS, 2011). Tetracycline is the predominant antibiotic used in veterinary medicine, accounting for 45% of antibiotics used in animals and equating to a UK national usage of approximately 174 tonnes of active substance a year (Figure 1.1A) (DEFRA, 2007). β -lactams are also important antimicrobials in veterinary treatment regimes, accounting for approximately 19% of antibiotics administered to animals in the UK (Figure 1.1A) (DEFRA, 2007). While commonly used cephalosporins used for human therapy are not used in the treatment of animals and vice-versa, many bacterial antibiotic resistance mechanisms confer a reduced susceptibility to both types of β -lactam (Li *et al.*, 2007). Therefore the use of veterinary licensed third and fourth generation cephalosporins such as ceftiofur and cefquinome respectively, may have implications for the prevalence of bacteria resistant to other clinically relevant third generation cephalosporins.

1.2.1 β -lactams

Due to their extensive use, β -lactam antibiotics have been described as the most important group of antimicrobials in both human and veterinary medicine (Li *et al.*, 2007; Llarrull *et al.*, 2011) (Figure 1.1). The molecules share the structural feature of a four membered β -lactam ring (Figure 1.2) and target enzymes known as penicillin binding proteins (PBPs) or peptidoglycan transpeptidases and transglycosylases (Sauvage *et al.*, 2008). By preventing these enzymes catalysing the cross-linking of

Figure 1.1. Common antibiotic classes used to treat humans and animals

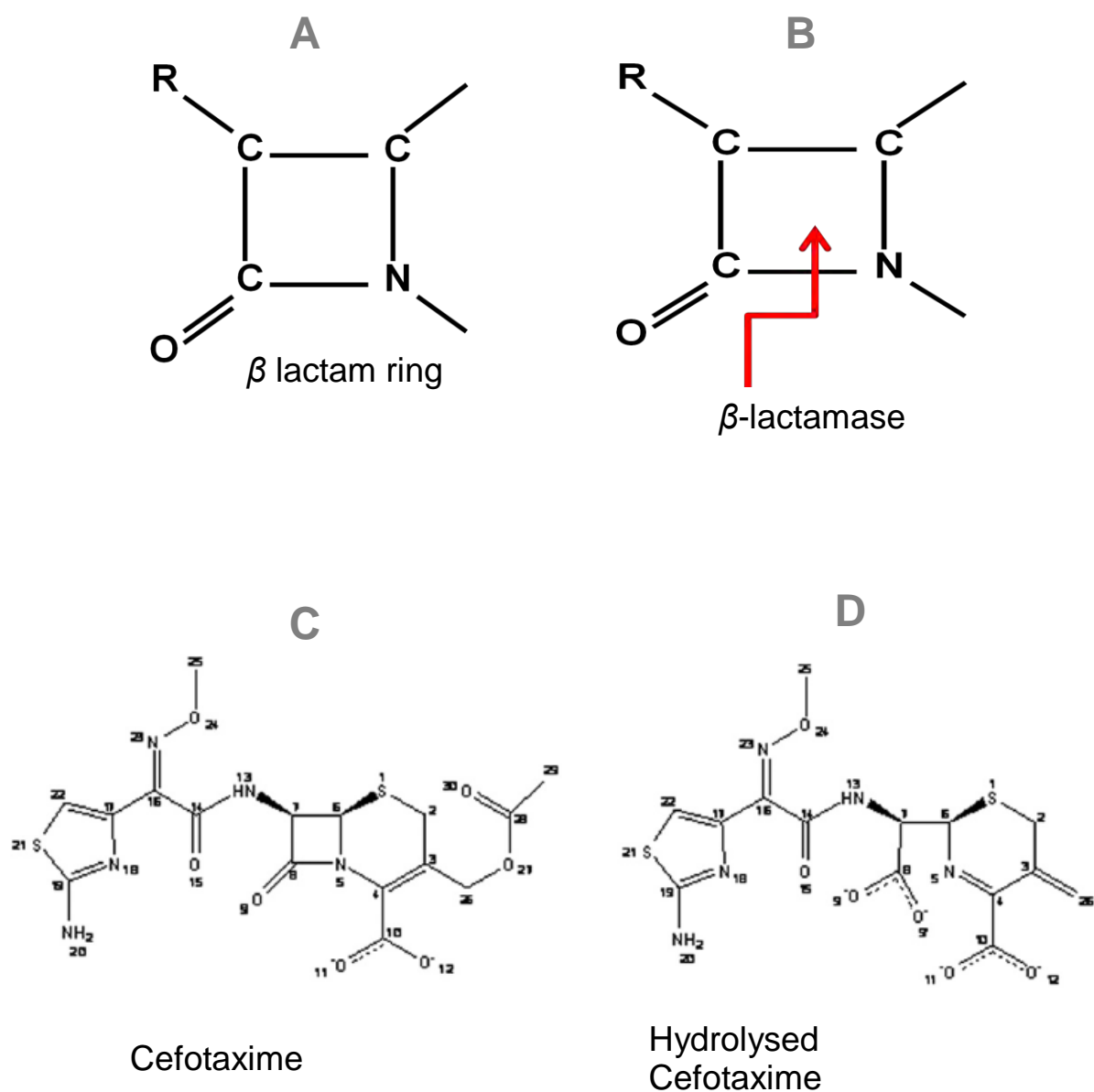


A, Trends in the number (in millions) of items of non-topical antibiotic prescribed clinically in England between October to September 1997-2009 (Adapted from NHS, 2011).

B, Tonnes of base active ingredient of each class of antimicrobial used in the UK in humans in community and hospital acquired infections, and used in animals in 2007 (Adapted from DEFRA, 2007).

Tetra, Tetracycline; T/S, Trimethoprim/Sulphonamide, Beta, β -lactams; Amino, Aminoglycosides; Macro, Macrolides; Quin, Quinolones.

Figure 1.2. The structure of β -lactam antibiotics and action of β -lactamases



- A) Structure of the β -lactam ring found in all β -lactam antibiotic,
 - B) The position where the β -lactam ring is hydrolysed by β -lactamases,
 - C) The chemical structure of cefotaxime,
 - D) The chemical structure of cefotaxime after hydrolysis by a CTX-M β -lactamase.
- (C and D adapted from Delmas *et al.*, 2010)

the peptidoglycan bacterial cell wall, rigidity of the structure is not achieved resulting in a bacteriostatic effect which can often result in bacterial cell death (Tipper, 1985).

1.2.2 Mechanisms of resistance to antibiotics

When antibiotics were first introduced in medicine over 60 years ago, bacteria with the ability to resist these antimicrobial effects were immediately discovered (Abraham, 1940; Abraham, 1987). Today, bacterial resistance to antibiotics is considered one of the most important contemporary healthcare issues; leading to an increase in treatment failure with adverse outcomes e.g. increased patient morbidity and mortality if an inappropriate antibiotic is administered (Hirsch and Tam, 2011; Shorr *et al.*, 2011). Antibiotic resistance also has dramatic cost implications such as the cost of isolation of patients and infection control (Pitout, 2008; Talbot, 2008); or loss of cheaper agents for effective treatment (Hawkey, 2008).

There are essentially four mechanisms by which bacteria may become resistant to antibiotic action: (i) modification of the antibiotic target, preventing successful interaction; (ii) metabolic bypass; (iii) reduction in the concentration of the drug within the cell either by reduced uptake or efflux of the antibiotic; and (iv) inactivation of the antibiotic, commonly mediated by the production of an enzyme (Cundliffe, 1989).

Both nosocomial and community acquired Gram negative bacterial infections are frequently treated with β -lactams (NHS, 2011); these bacteria are commonly resistant to antimicrobials (Pitout, 2008). Resistance to β -lactams is mediated by four mechanisms; restriction of antimicrobial concentration within the bacterial cell (e.g. via porin loss or active efflux); a decrease in affinity of the β -lactam for the target PBP enzymes; or hydrolysis of the drug by a β -lactamase enzyme (Figure 1.2) (Piddock *et al.*, 1997; Pfeifer *et al.*, 2010). The latter of these mechanisms is by far the most

common in *Enterobacteriaceae* (Rodriguez-Bano and Pascual, 2008; Bush and Jacoby, 2010) due in part to the dissemination of these genes by mobile genetic elements.

1.2.2.1 *Mobilisation of antibiotic resistance genes*

There are several elements that are able to mobilise antibiotic resistance genes from one genetic location to another. These include (i) bacteriophages or 'phage', which may excise extra DNA located adjacent to integrated prophage in one bacterium, and integrate this genetic material within the chromosome of another on re-infection (Brussow *et al.*, 2004); (ii) insertion elements and transposons, short DNA sequences able to translocate genes using an enzyme 'transposase' which allows excision and integration of genetic material using homologous recombination (Normark and Normark, 2002); (iii) conjugative transposons which go a step further and allow the excised mobilised genes to transfer to another bacterium and integrate within the chromosome via horizontal transfer (Slater *et al.*, 2008) and (iv) plasmids, some of which allow the translocation of genetic material from one host cell to another via conjugation (Section 1.4). Other genetic elements, such as integrons, are able to recognise and capture genetic material through an integrase protein, however can only mobilise accumulated DNA when associated with one of the genetic elements listed above (Fluit and Schmitz, 2004).

1.3 β -lactamases

β -lactamases are enzymes produced by many bacteria, thought to be an evolutionary response to β -lactam antibiotics synthesised by environmental organisms within soil (Bradford, 2001). These enzymes achieve inactivation of β -lactam antibiotics by

cleavage of the amide bond resulting in hydrolysis of the β -lactam ring (Figure 1.2) (Bush *et al.*, 1995; Bonnet, 2004). β -lactamases are structurally similar to PBPs and are postulated to have evolved either from these cell-wall enzymes or to share a common ancestor (Joris *et al.*, 1988; Massova and Mobashery, 1998). β -lactamases were first recognised in the form of penicillinases, which were able to hydrolyse the very first β -lactam benzyl penicillins used to treat Gram-positive bacterial infections (Abraham, 1940). In response, pharmaceutical companies produced penicillinase stable antibiotics such as methicillin and flucloxacillin. Within one year of use in patients, β -lactamases able to hydrolyse these new drugs were also found in clinical isolates (Jevons, 1961). The treatment of Gram negative infections was revolutionised by the introduction of semi-synthetic penicillins such as ampicillin, which have a β -lactam ring attached to a synthetic side chain and so increased activity against the *Enterobacteriaceae* (Rolinson, 1998). Unfortunately, ampicillin-resistant Gram-negative bacteria were quickly isolated and shown to carry a β -lactamase (*bla*_{TEM} gene) on a plasmid (Bradford, 2001). More sophisticated β -lactam drugs were then developed. These compounds could resist hydrolysis from both TEM and also SHV β -lactamases; these agents were named third generation cephalosporins or oxyimino-cephalosporins. The third generation cephalosporins have a very broad spectrum of activity and are used extensively in human medicine and to a lesser extent in veterinary practice (Goossens *et al.*, 2005; Li *et al.*, 2007).

Mobilisation of novel β -lactamase genes from environmental bacteria and evolution of *bla*_{TEM} and *bla*_{SHV} genes gave rise to enzymes able to inactivate these newer cephalosporins. This group of enzymes were collectively named the extended spectrum β -lactamases or 'ESBLs'. Plasmid mediated SHV β -lactamases are thought

to have originated from several 'escapes' of the chromosomal *bla*_{SHV} gene of *Klebsiella pneumoniae* (Gniadkowski, 2008). Point mutations in this gene have led to enzymes with extended substrate spectrums. The origins of plasmid based *bla*_{TEM} genes are currently unknown, however, they are postulated to have originated from a chromosomal progenitor (Livermore, 2008).

More recently a new group of β -lactamase enzymes called carbapenemases (Table 1.1; groups 2c, 2df and 3) have also emerged, which are able to hydrolyse the widely used and often last resort carbapenem antibiotics (Walsh, 2008; Bush, 2010).

1.3.1 Classification of β -lactamases

Many β -lactamase classification systems have been adopted in order to categorise these enzymes. However, as more diverse enzymes are discovered, and more is learned about their phylogeny, these systems have increased in complexity.

The most commonly used classifications of β -lactamases are by Ambler (Groups A-D) based on molecular structure and amino acid sequence (Ambler *et al.*, 1991), and by Richmond and Sykes (Richmond and Sykes, 1973). Richmond and Sykes classify β -lactamases based on their clinical functional properties, whether they are inhibited by β -lactamase inhibitors such as clavulanic acid and the drugs which are preferentially hydrolysed. The Bush Jacoby and Medeiros classification combines both of these systems (Bush *et al.*, 1995) and has been updated to include recently discovered enzymes (Bush and Jacoby, 2010) allowing better differentiation between groups (Table 1.1). Fundamentally, β -lactamases can be divided between those enzymes which contain a serine active site residue (Ambler classes A, C and D) and those with a catalytic zinc centre called metallo β -lactamases (Ambler class B). The serine containing enzymes can be further subdivided into those that are inhibited by

Table 1.1. β -lactamase classification (adapted from Bush, 2010 and Pfeifer *et. al.*, 2010)

| Active site residue | Ambler class | Group* | β -lactamases | Exemplars | Common host species | Agents inactivated | Inhibited by clavulanic acid |
|---------------------|--------------|--------|---------------------------------------|--|--|---|------------------------------|
| 11 Serine | A | 2a | Penicillinases | | <i>Staphylococcus aureus</i> | Penicillin | ✓ |
| | | 2b | Penicillinases and cephalosporinases | TEM-1, TEM-2, SHV-1, SHV-11 | <i>Enterobacteriaceae</i> | Ampicillin | ✓ |
| | | 2be | Extended spectrum beta-lactamases | TEM-3 – TEM167 SHV-2 – 117 All CTX-Ms PER/VER/GES | <i>Enterobacteriaceae</i> | Penicillins and 3 rd generation cephalosporins | ✓ |
| | | 2br | Inhibitor resistant penicillinases | TEM-30 – 41, 44, 45, 51 and 54; SHV-10 | <i>Enterobacteriaceae</i> | Penicillins | Reduced |
| | | 2c | Carbapenemases | PSE/NMC/IMI/KPA-1/2/3 GES-2 and SHV-38 SME/KPC | <i>Enterobacteriaceae</i> | Penicillins and carbapenems | ✓ |
| | | 2e/f | Cephalosporinases | | <i>Enterobacteriaceae</i> | Cephalosporins /monobactams | ✓ |
| | C | 1 | AmpC cephamycinases (chromosomal) | AmpC | <i>Enterobacter spp.</i> <i>Citrobacter spp.</i> | Cefoxitin/ 3 rd gen. cephalosporins | X |
| | D | 2d | AmpC cephamycinases (plasmid encoded) | CMY, DHA, MOX, FOX, ACC | <i>Enterobacteriaceae</i> | Cefoxitin and 3 rd generation cephalosporins | ✓ |
| | | | Broad spectrum β -lactamases | OXA-1, OXA-9 | <i>Enterobacteriaceae</i> , <i>A. baumannii</i> | Oxacillin, ampicillin | ✓ |
| | | | ESBL OXA | | <i>Enterobacteriaceae</i> , <i>A. baumannii</i> | Penicillin and 3 rd generation cephalosporins | ✓ |
| | | 2df | Carbapenemases | OXA-48, OXA-23, 24,25 | <i>Enterobacteriaceae</i> , <i>A. baumannii</i> | Carbapenems | ✓ |
| Metallo | B | 3 | Carbapenemases | VIM, IMP, NDM-1 | <i>Enterobacteriaceae</i> , <i>P. aeruginosa</i> , <i>A. baumannii</i> | All β -lactams except monobactams | X |

*Bush Jacoby and Medeiros classification. TEM, Temoniera; SHV, sulfhydryl variant; CTX-M, cefotaximase - Munich; PER, P. Nordmann, E. Ronco R. Labia; VER, Versailles; GES, Guiana extended spectrum; PSE, *Pseudomonas*-specific enzymes; NMC/IMI, not metalloenzyme carbapenemase/imipenem-hydrolysing beta; KPA, *Klebsiella pneumoniae* group A; SME, *Serratia marcescens* enzyme; KPC, *K. pneumoniae* carbapenemase; OXA, oxacillinase; CMY, cephamycinases; DHA, Dhahran hospital Saudi Arabia; MOX, moxalactamase; FOX, cefoxitinase; ACC, Ambler class C; VIM, Verona integron encoded metallo; IMP, imipenemase; NDM, New-Delhi Metallo.

clavulanic acid and other β -lactamase inhibitors (Ambler classes A and D) and those that are not (Ambler class C, group 1) (Bush and Jacoby, 2010). Groups A and D are further distinguished by their difference in amino acid and DNA sequence and disparity in activity to different substrates (Hall and Barlow, 2004).

1.3.2 Extended spectrum β -lactamases (ESBLs) – Ambler class A/ Group 2be

The term 'extended broad spectrum β -lactamases' was first applied in 1987 to TEM and SHV enzyme variants with the ability to hydrolyse cephalosporins with a C7 side chain containing an oxyimino group (Jarlier *et al.*, 1988). These enzymes are thought to have derived from *bla*_{TEM-1/2} and *bla*_{SHV-1} β -lactamase genes, which acquired point mutations within the DNA sequence encoding the active-site of the enzyme. This allowed a physical expansion of the active site and so a broadening of the range of substrates these enzymes are able to hydrolyse (Gniadkowski, 2008). However, the mutations conferred an enzyme with a reduced catalytic efficiency compared with the 'parental' enzymes against penicillins, and gave increased susceptibility to β -lactamase inhibitors (Bradford, 2001).

Since the late 1980s, other classes of β -lactamases derived from enzymes other than TEM/SHVs have been discovered; these also have activity against the oxyimino-cephalosporins. As a result the term 'ESBL' was extended to include these other enzymes (Livermore, 2008). Currently, an ESBL is defined as any β -lactamase capable of inactivating penicillins, first, second and third generation cephalosporins and aztreonam; but not cephamycins or carbapenems. These enzymes are inhibited by β -lactamase inhibitors such as amoxicillin-clavulanate, tazobactam or sulbactam (Paterson and Bonomo, 2005).

Following the appearance of TEM/SHV ESBL variants in the mid 1980s, these enzymes spread globally in the 1990s (Cantón and Coque, 2006). Both TEM and SHV groups expanded in the number of variants identified, and became a significant clinical treatment issue causing increased mortality as a result of treatment failure (Miro *et al.*, 2005). Risk factors for acquiring an infection with a bacterium producing a TEM or SHV ESBL include admittance to ICU; recent surgery or long term hospital stay; placement of a catheter and previous cephalosporin treatment. Therefore, infections with strains carrying TEM/SHV enzymes have been largely nosocomial related (Canton *et al.*, 2008).

The dominance of these enzymes has been succeeded by the dramatic emergence of another group of ESBLs named the CTX-Ms which have spread rapidly since 1995. These are now the most widespread and diverse group of ESBLs isolated worldwide (Bonnet, 2004; Hawkey and Jones, 2009).

1.3.3 CTX-M β -lactamases

The first CTX-M β -lactamase was identified in Japan in 1986 during a pharmacokinetic study of β -lactam antibiotics in a dog. The enzyme was identified in an isolate from a stool sample, and named FEC-1 (Matsumoto *et al.*, 1988). It was not until 1989 when Bauernfeind *et al.*, (1990) reported a non TEM/SHV β -lactamase producing isolate resistant to cefotaxime, that the group acquired its name 'CTX-Mases' or 'CTX-Ms' with reference to the preferential hydrolytic activity of members for cefotaxime (Lartigue *et al.*, 2004) and the location of the group, 'Munich'. This enzyme was named CTX-M-1 and was later assigned to 'Group 1' when it became apparent CTX-Ms could be sub-categorised based on amino acid sequences.

Simultaneously, cefotaxime resistant *Salmonella* Typhimurium strains began disseminating in Argentina (Bauernfeind *et al.*, 1992).

In 1992, Bernard *et al.* reported an *E. coli* isolated in 1989 which was resistant to cefotaxime; the enzyme responsible was cloned and named MEN-1. Sequence analysis of *bla*_{MEN-1} showed 39% identity with *bla*_{TEM} and *bla*_{SHV} variants (Barthelemy *et al.*, 1992). Later analysis and DNA sequencing showed that *bla*_{MEN-1} and *bla*_{CTX-M-1} were identical, and that *bla*_{Toho-1} sequenced in Japan (Ishii *et al.*, 1995) was of the same genotype (renamed *bla*_{CTX-M-2}). FEC-1 was also found to be identical to an enzyme found from an isolate from Poland named CTX-M-3 in 1996 (Gniadkowski *et al.*, 1998).

Since 1998, the CTX-M family has continued to increase rapidly (Navarro and Miro, 2002) with 117 different variants currently recognised

(<http://www.lahey.org/studies/webt.htm>; as of September 2011). Almost all isolates found to produce these β -lactamases are *Enterobacteriaceae* such as *E. coli* (found most frequently), *Salmonella* spp., *Klebsiella* spp., *Shigella* spp., *Citrobacter* spp., *Enterobacter* spp. and *Proteus* spp. However, CTX-M producing *Pseudomonas* spp., *Vibrio cholerae* El Tor (Petroni *et al.*, 2002), *Serratia marcescens* (Mlynarczyk *et al.*, 2009), *Acinetobacter baumannii* (Radice *et al.*, 2002), *Aeromonas hydrophilia*, *Providencia stuartii*, *Burkholderia cepacia*, *Stenotrophomonas maltophilia* (Kiratisin and Henprasert, 2010) and *Morganella morganii* (Ogbolu *et al.*, 2011; Soleimanian *et al.*, 2011) have also been reported.

1.3.3.1 Activity and structure of CTX-M β -lactamases

The CTX-M enzymes are characterised by their preferential hydrolysis of cefotaxime over ceftazidime, in most cases this activity is up to 35 times greater (Bonnet, 2004).

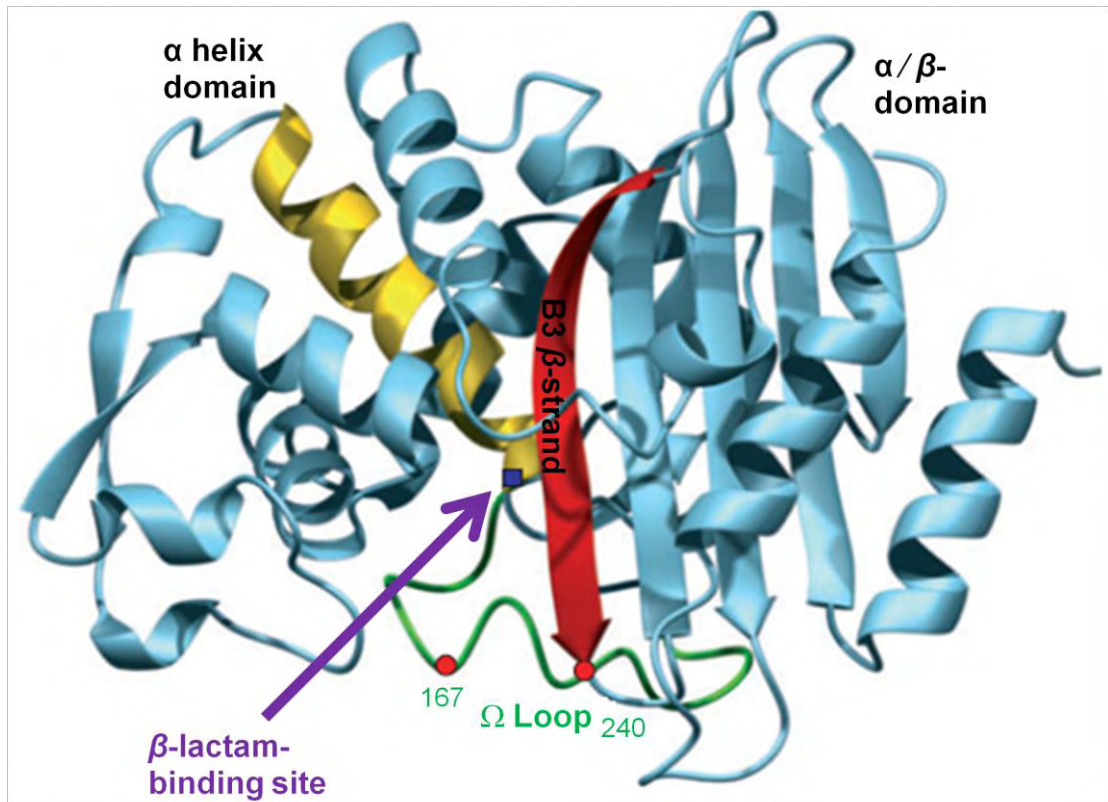
As a group they are inhibited by tazobactam more efficiently than by sulbactam and amoxicillin-clavulanate (Paterson and Bonomo, 2005), and have reduced activity against penicillin compared with TEM or SHV variants (Lartigue *et al.*, 2004). The molecular structure of these enzymes comprises an α helical domain and a mixed α helices / β sheet domain, with the active β -lactam binding site between them (Figure 1.3).

The majority of CTX-M enzymes contain 291 amino acid residues and have a molecular mass of ~28 kDa (Bonnet, 2004). These enzymes also contain an Ω loop near the active site (Navarro and Miro, 2002). The DNA sequence encoding this locus is where mutations may lead to enhanced activity against ceftazidime; such as mutations seen in *bla*_{CTX-M-15}, *bla*_{CTX-M-16}, *bla*_{CTX-M-19} and *bla*_{CTX-M-27} (Bonnet *et al.*, 2001; Karim *et al.*, 2001; Bonnet, 2004). *bla*_{CTX-M-15} is thought to be the result of a mutated *bla*_{CTX-M-3} gene which has a Gly-240-Asp substitution leading to an eight fold higher action against ceftazidime (Poirel *et al.*, 2001). The 117 identified CTX-M variants cluster into five different phylogenetic groups based on their amino acid sequences. Each group is named after the first enzyme identified within this cluster (Groups 1, 2, 8, 9 and 25). Within groups, each member shares >95% amino acid homology and are thought to have a common ancestor; whereas members belonging to distinct groups share <90% identity (Bonnet, 2004).

1.3.3.2 Origins of *bla*_{CTX-M}

Unlike *bla*_{TEM} and *bla*_{SHV} gene, *bla*_{CTX-M} β -lactamase genes are thought to have progenitors with an innate ability to hydrolyse oxyamino-cephalosporins (Hawkey, 2008). These *bla*_{CTX-M} progenitor chromosomal genes can be found in the genus *Kluyvera*, an environmental *Enterobacteriaceae* bacterium predominantly found in

Figure 1.3. Ribbon diagram of CTX-M-9



As with all serine β -lactamases the molecular fold consists of an α -helical domain (left) and a mixed α/β -domain (right). The serine β -lactam-binding site (Shown by the purple arrow) is located in a cleft between the two domains. Positions 167 and 240, where amino-acid substitutions enhancing ceftazidimase activity occur, are located either in the Ω -loop (in green, at the bottom of the binding site) or in the terminal part of the B3 β -strand.

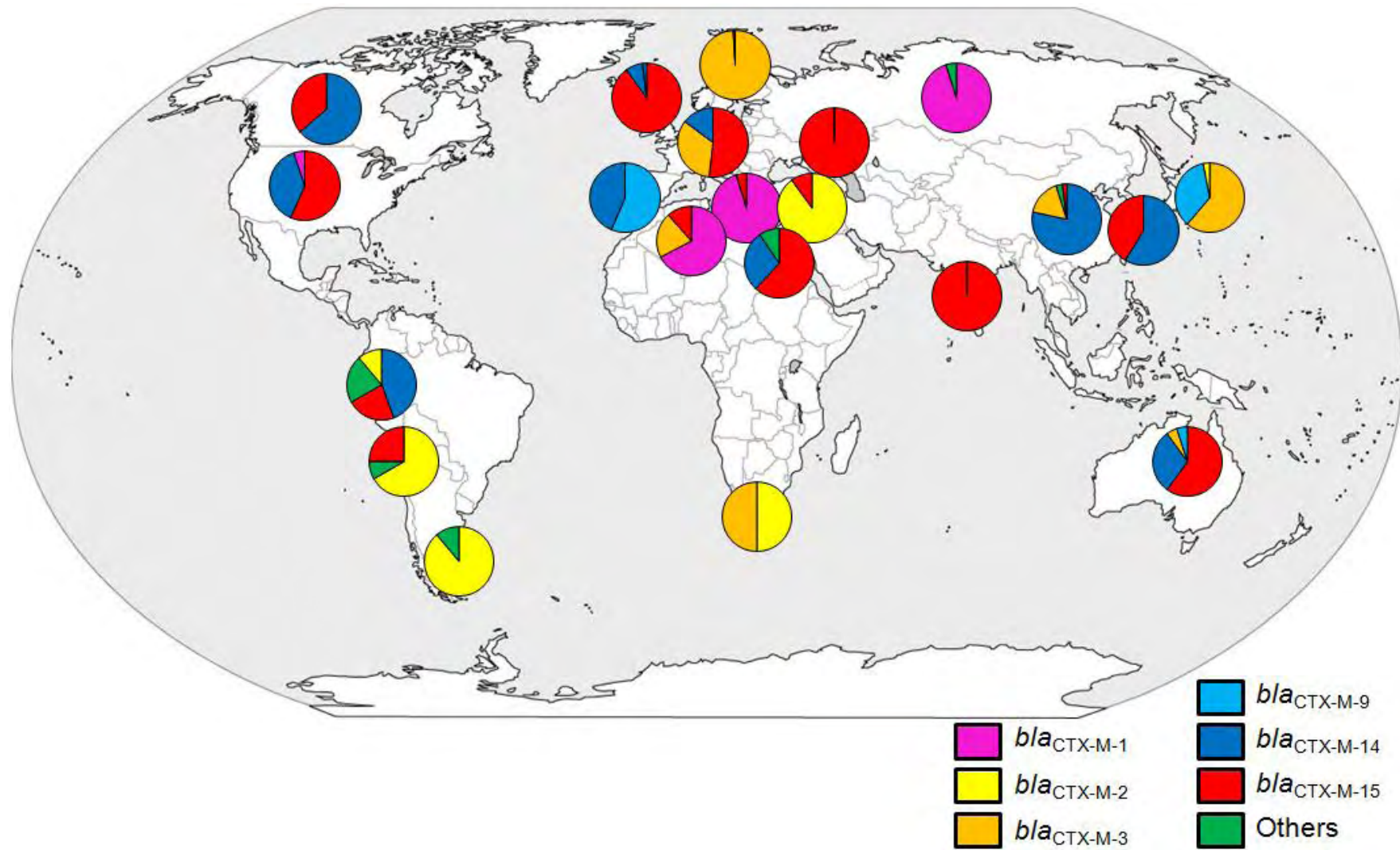
Adapted from Rossolini *et al.*, (2008).

soil, water sewage or food products of animal origin such as milk (Farmer *et al.*, 1981; Pavan *et al.*, 2005). *Kluyvera* spp. can also be found as part of the human gut flora (associated with low total bacterial counts) but are rarely reported from clinical specimens (Farmer *et al.*, 1981; Sarria *et al.*, 2001). Phylogenetic analysis of the *Kluyvera* β -lactamase genes have shown that mobilisation of these different *Kluyvera* genes may have led to the various groups of *bla*_{CTX-M}. For example, β -lactamase genes from *K. ascorbata* (*bla*_{KLUA}) share 99% identity with those members of CTX-M Group 1 and 2 (Rodriguez *et al.*, 2004), *K. georgiana* *bla*_{KLUY} genes show 100% DNA homology with *bla*_{CTX-M} Group 9 gene *bla*_{CTX-M-14} (Olson *et al.*, 2005) and to a lesser extent *K. cryocrescens* gene *bla*_{KLUC-1} and *K. georgiana* *bla*_{KLUG-1} have 99% amino acid homology with *bla*_{CTX-M-8} (Poirel *et al.*, 2002). The origins of *bla*_{CTX-M} groups 25 and 45 are unknown, but are also thought to have originated from *Kluyvera* spp. (Rossolini *et al.*, 2008). Within the recent literature CTX-M-14 and CTX-M-3 are the accepted progenitors of their respective groups (Group-9 and Group-1). This suggests subsequent diversification from these progenitor variants has led to the evolution of the other *bla*_{CTX-M} alleles within these groups (Novais *et al.*, 2010).

1.3.3.3 Epidemiology of *bla*_{CTX-Ms}

In different geographical areas of the world, the predominant *bla*_{CTX-M} genes identified in CTX-M producing isolates varies considerably (Canton *et al.*, 2008; Hawkey and Jones, 2009). The global distribution of CTX-M variants is shown in Figure 1.4. There are currently no plausible explanations for variation across global regions.

Figure 1.4. Global distribution of *bla*_{CTX-M} variants and their relative proportions within each region



Adapted from Hawkey and Jones (2009)

The first CTX-M producing bacterium identified in the UK was isolated from a patient in Leeds in 2000 (Alobwede *et al.*, 2003), produced by a *Klebsiella oxytoca* carrying a *bla*_{CTX-M-9} gene. This was followed in 2001-2002 by an outbreak in Birmingham caused by a CTX-M-25 producing strain of *K. pneumoniae* (Brenwald *et al.*, 2003). By 2003, when Munday *et al.*, conducted a study on isolates from York identifying prevalent ESBL types, CTX-M producers were common. They found *bla*_{CTX-M} variants *bla*_{CTX-M-9}, *bla*_{CTX-M-14} and *bla*_{CTX-M-15} during the study, however failed to detect any CTX-M producing isolates in a collection of earlier strains isolated between 1990-1991 (Piddock *et al.*, 1997; Munday *et al.*, 2004). At present, in the UK a large range of CTX-M enzymes are produced by bacteria including the only representative of Group 8, CTX-M-40 (Hopkins *et al.*, 2006), CTX-M-25, 26, 9, 1, 3, 10, 14 and 15 (Munday *et al.*, 2004). CTX-M-15 β -lactamase producing bacteria are the most commonly isolated in the UK (Rossolini *et al.*, 2008), with CTX-M-14 the second most prevalent (Warren *et al.*, 2008). In 2010, 10% of *E. coli* isolated from human blood cultures were resistant to cephalosporins compared with 6% in 2004, indicating the significant problem these isolates are causing in the UK (HPA, 2011).

1.3.3.4 Clonal expansion of strains carrying *bla*_{CTX-M5}

The global spread of *bla*_{CTX-M-15}, currently the most commonly identified ESBL worldwide, can be partly attributed to the extensive clonal expansion of one particular *E. coli* sequence type, ST131, with which a selection of *bla*_{CTX-M-15} carrying plasmids have become associated (Lau *et al.*, 2008; Clermont *et al.*, 2009; Woodford *et al.*, 2009). *E. coli* B2-O25:H4-ST131 is primarily an extraintestinal pathogenic *E. coli* and a member of the highly virulent B2 *E. coli* phylogenetic subgroup 1 found globally isolated from humans, and more recently in companion and human associated

animals such as rats (Ewers *et al.*, 2010; Guenther *et al.*, 2010; Rogers *et al.*, 2011). Typically these isolates are multidrug resistant, demonstrating resistance to ciprofloxacin, gentamicin, tobramycin and tazobactam and many harbour plasmids carrying *bla*_{OXA-1}, *bla*_{TEM-1} and the quinolone resistance gene *aac(6')-Ib-cr* in addition to *bla*_{CTX-M-15} (Pitout *et al.*, 2009). Initially, an association was made between *E. coli* ST131 hosts and *bla*_{CTX-M-15} carrying IncFII plasmids, however since 2008, ST131 isolates with plasmids carrying many other *bla*_{CTX-M} variants have been reported (Clermont *et al.*, 2009; Oteo *et al.*, 2010), including *bla*_{CTX-M-2}, *bla*_{CTX-M-3}, *bla*_{CTX-M-27} (Ruppe *et al.*, 2009), *bla*_{CTX-M-32} and *bla*_{CTX-M-14}. It appears that ST131 CTX-M-14 producing strains are emerging in the geographical regions where *bla*_{CTX-M-14} is most commonly identified, such as Spain (Blanco *et al.*, 2009; Mora *et al.*, 2010) and Asia. Although a CTX-M-14 producing O25:H4-ST131 strain was the cause of an *E. coli* outbreak in a Japanese hospital in 2002 (Suzuki *et al.*, 2009), there is still no evidence to date that CTX-M-14 ST131 producing strains are spreading via clonal expansion.

1.3.3.5 Acquisition, carriage and reservoirs of CTX-M producing bacteria

Until the end of the 1990s, infections by bacteria producing an ESBL were most commonly associated with hospital acquired infections particularly in the ICU and after surgical procedures or after the use of catheters (Paterson and Bonomo, 2005). Infections were predominately caused by isolates of *Klebsiella spp.* expressing *bla*_{TEM} or *bla*_{SHV} genes, and were associated with prior use of a cephalosporin (Canton *et al.*, 2008). Since this time ESBL epidemiology has changed dramatically with the rapid emergence of CTX-M producing isolates. Whereas infections with TEM or SHV ESBL producers are largely nosocomial, CTX-M producing isolates have frequently

been identified in *E. coli* from the community (Woodford *et al.*, 2004; Rodriguez-Bano *et al.*, 2010), and are the most commonly isolated ESBLs from urinary tract pathogens (Pitout *et al.*, 2004). Health-care settings, such as nursing homes, have also been strongly associated with CTX-M acquisition (Dhanji *et al.*, 2010; March *et al.*, 2010; Schoevaerdts *et al.*, 2011), either resulting in infections or carriage of these isolates (Rooney *et al.*, 2009). Despite a clear link between age (>65s) and carriage (Schoevaerdts *et al.*, 2011), healthy people of all ages have been identified as carrying CTX-M producing bacterial strains (Tian *et al.*, 2008; Sasaki *et al.*, 2010) which are able to pass to others, particularly between co-habitors and even family pets (Johnson *et al.*, 2008; Lo *et al.*, 2010).

Increased foreign travel is also believed to be a major contributor to the emergence and spread of *bla*_{CTX-M} variants (Laupland *et al.*, 2008; Pitout *et al.*, 2009). Tängdén *et al.* (2010) showed 24/100 healthy volunteers who travelled to locations outside northern Europe including Africa, Asia, India, the Middle East and Southern Europe, returned with a previously undetected colonisation of a CTX-M producing isolate. It is not known whether these travel related acquisitions are due to colonisation by the *E. coli* isolates themselves or integration of *bla*_{CTX-M} carrying plasmids within the host's resident flora.

Other potential reservoirs where CTX-M producers can be found include soils (particularly when enriched using manure) (Heuer *et al.*, 2008), sludge and sewage (Reinthaler *et al.*, 2009) and water sources such as rivers (Chigor, 2010; Dhanji *et al.*, 2010). These environmental reservoirs may have constant or fluctuating exposure to many different antibiotics and biocides and are composed of diverse bacterial populations and nutrient sources (Davies and Davies, 2010). Their role in

persistence, amplification, evolution and spread of resistance genes is currently still undetermined, but may be particularly relevant in the acquisition of *bla*_{CTX-Ms} by bacteria isolated from animals.

1.3.3.6 CTX-M producing bacteria from animals

As previously described, the β -lactam antibiotics are an important group of antimicrobials in veterinary medicine (Li *et al.*, 2007) (Figure 1.1). β -lactams are used outside the EU as a prophylactic in animal feed, in aquaculture and were previously used at sub-therapeutic levels to improve growth rates and feed efficiency in food animals (Aarestrup, 2004; Davies and Davies, 2010). It is estimated that the US uses more than 8000 tonnes of antimicrobials annually in food production and that the UK farming industry uses three tonnes of cephalosporins for therapeutic purposes alone each year (Carattoli, 2008); therefore exposure of food animals to these compounds is very high. Despite this, ESBL producing bacteria are much less frequently isolated from animals than humans, and strains prevalent in food animals in a given geographical area do not necessarily reflect the strains found in human populations (Carattoli, 2008; Hunter *et al.*, 2010). Recently, *bla*_{CTX-M} carrying strains have become the most commonly detected ESBL producing bacteria isolated from animals (Li *et al.*, 2007). CTX-M producers have been identified globally in poultry (Brinas *et al.*, 2003; Dierikx *et al.*, 2010; Sheldon, 2010), swine (Escudero *et al.*, 2009; Smet *et al.*, 2009) and cattle populations (Liebana *et al.*, 2006; Hunter *et al.*, 2010) and have been isolated in poultry farms with no known exposure to β -lactam antibiotics (Smet *et al.*, 2008; Bortolaia *et al.*, 2010).

β -lactam resistant *E. coli* can also be isolated from companion animals such as cats and dogs (Moreno *et al.*, 2008; Ewers *et al.*, 2010) and from wild animals such as

foxes, kestrels and sparrow-hawks (Costa *et al.*, 2006; Costa *et al.*, 2008). Currently, domesticated pets are thought to have higher carriage rates of resistant strains than livestock and due to their close contact with humans, pets may create an important ecological reservoir for ESBL producing bacteria (Skurnik *et al.*, 2006). Therefore, the contamination of food products such as meat, dairy or eggs, and colonisation of flora of animal intestinal tracts may provide a reservoir of *bla*_{CTX-Ms} (Warren *et al.*, 2008), and a diverse environment for the transmission of plasmids carrying resistance genes to humans (Poppe *et al.*, 2005; Ensor *et al.*, 2006; Skurnik *et al.*, 2006).

Animals, particularly livestock, have historically been considered as reservoirs for antibiotic resistant bacteria and plasmids (Hartley *et al.*, 1975; Timoney and Linton, 1982; Hinton and Linton, 1983). However, the spread of these isolates and genetic elements from animals to humans or vice-versa has remained controversial, shrouded in political and economic issues. A body of evidence now exists showing transfer of either particular bacterial strains or plasmids between these hosts and environments; however the mechanisms and implications of this dissemination are largely unknown (Mulvey *et al.*, 2009; Smet *et al.*, 2009).

1.3.4 CTX-M-14

CTX-M-14, also known as CTX-M-18, UOE-2 and Toho-3 (Navarro and Miro, 2002) has become the second most common CTX-M enzyme isolated worldwide (Hawkey and Jones, 2009; Song *et al.*, 2009; Furtado and Nicolau, 2010). A member of Group 9, CTX-M-14 has only one amino acid difference from CTX-M-9 with a change of alanine to valine at position 231 (Ma *et al.*, 2002). Analysis of the crystal structure of CTX-M-14 indicates these substitutions have led to specific interactions between enzyme and substrate which may be responsible for the improved activity of the

enzyme against some β -lactams, rather than active site expansion (Perez *et al.*, 2007) as seen in TEM and SHV variants. However, CTX-M-14 retains the characteristics of a classic CTX-M β -lactamase, and does not have the ability to hydrolyse ceftazidime.

1.3.4.1 *Emergence and epidemiology*

CTX-M-14 was first identified from an *E. coli* isolated from a patient in 1998 in China (Chanawong *et al.*, 2002). The sequence was elucidated and deposited in Genbank as accession number AF252621.1 (now updated as AF252621.2) and was quickly followed by the detection of this enzyme in other parts of Asia, including Korea (the first published CTX-M-14 producer isolated in 1995) and in Japan (Pai *et al.*, 2001). During the late 1990s more reports of *bla*_{CTX-M-14} came from China, France, Taiwan and Brazil (Bou *et al.*, 2002; Dutour *et al.*, 2002; Yu *et al.*, 2002). In 2001, a CTX-M-14 producing *E. coli* isolate was identified in Spain (Bou *et al.*, 2002) and a *Salmonella* Enteritidis CTX-M-14 producer was isolated in the UK (Hopkins *et al.*, 2006). Since this time, a rapid increase in the number of CTX-M-14 producers has been observed worldwide (Figure 1.4) (Cao *et al.*, 2002; Chanawong *et al.*, 2002; Pallecchi *et al.*, 2007; Bae *et al.*, 2008; Lee *et al.*, 2009) becoming the most prevalent ESBL in a number of countries including Spain and China. In the UK, CTX-M-14 has become the second most prevalent CTX-M enzyme type identified in ~10% of clinical ESBL producing *E. coli* (HPA, 2011). All CTX-M-14 producing isolates documented to date have been members of the family *Enterobacteriaceae* and in a wide variety of species within this group.

1.3.4.2 *bla*_{CTX-M-14} mobilisation

As previously described, *bla*_{CTX-M} Group 9 genes found on plasmids are thought to have originated from chromosomal *K. georgiana* β -lactamase gene *bla*_{KLUY-1}, which shares 100% amino acid identity with group 9 progenitor, *bla*_{CTX-M-14} (Olson *et al.*, 2005). A 42 base-pair upstream region of *bla*_{CTX-M-14} is identical to those found upstream of *bla*_{KLUY-1}; however *bla*_{CTX-M-14} is also found downstream of insertion element *ISEcp1* not found in the *Kluyvera* chromosome.

ISEcp1 belongs to the IS1380 insertion sequence family (Lartigue *et al.*, 2006), consisting primarily of two imperfect inverted repeats and an open reading frame encoding a 420 amino acid transposase (Lartigue *et al.*, 2004). In all plasmids containing *bla*_{CTX-M-14} investigated to date, all or part of this element has been found upstream of the *bla*_{CTX-M-14} gene (Eckert *et al.*, 2006) and *ISEcp1* can also be found upstream of *bla*_{CTX-M-3}, *bla*_{CTX-M-10} and *bla*_{CTX-M-15} (Lartigue *et al.*, 2004). It is believed this element has a major role in mobilisation of the *bla*_{CTX-M} genes. Lartigue *et al.* (2006) demonstrated that *bla*_{CTX-M-2} could be mobilised *in vivo* from its natural progenitor *K. ascorbata* to a plasmid when *ISEcp1B* was placed upstream of this gene; however the reason for its strong association with *bla*_{CTX-M} genes is still unknown (Poirel *et al.*, 2005). *bla*_{KLUY-1} is very weakly expressed on the *Kluyvera* chromosome, therefore it is possible that this foreign insertion sequence acts as a strong promoter to give *bla*_{CTX-M-14} its characteristic phenotype when present on plasmids. However, no chromosomal origin or reservoir of *ISEcp1* has yet been found (Poirel *et al.*, 2003). Another insertion element, IS903, has been detected downstream of all studied *bla*_{CTX-M-14S}, however is not believed to have a role in mobilisation of this gene (Poirel *et al.*, 2005).

1.4 Plasmids

Plasmids are extra-chromosomal circular fragments of DNA that replicate autonomously in host cells (Clowes, 1972). They are ubiquitous in most bacterial species and are found in virtually all ecosystems (Slater *et al.*, 2008) including hydrothermal vents (Prieur *et al.*, 2004) and arctic soil (Duodu *et al.*, 2007). Plasmids encode genes essential for the initiation and control of their own replication and can encode a variety of traits that in certain circumstances may be advantageous to the host bacterial cell such as antibiotic resistance, heavy metal resistance or may carry genes for adaption and survival in particular environments (Johnson *et al.*, 2007). Therefore, plasmids are believed to play a major role in bacterial adaption to environmental change and contribute to overall bacterial genome plasticity. Plasmids are also important when carried by medically relevant bacteria due to their ability to mobilise and disseminate antibiotic resistance genes and virulence genes, which may impact on the pathogenicity of a bacterium. Of note in relation to this study, is their role in the dissemination of genetic material encoding β -lactamase enzymes among Gram-negative *Enterobacteriaceae*.

1.4.1 Characterisation of plasmids

Naturally occurring plasmids are notoriously difficult to categorise due to their mosaic nature, propensity to recombine and the lack of any universally common genetic features (Coque *et al.*, 2008; Call *et al.*, 2009; Villa *et al.*, 2010). However, a need for a common nomenclature and a grouping system to allow epidemiological and evolutionary studies stimulated the development of both phenotypic and subsequent genotypic schemes (Novick *et al.*, 1976).

1.4.1.1 *Incompatibility and rep typing*

The first and most widely recognised typing system is based on the phenotypic observation that two plasmids sharing common replication and partitioning elements are unable to stably proliferate within the same bacterial cell (Couturier *et al.*, 1988). Classically, membership of an incompatibility group (Inc group) was ascertained by the introduction, by conjugation or transformation, of the candidate plasmid into a strain carrying a plasmid of known Inc type. If the resident plasmid was eliminated within the progeny the test plasmid would be assigned to this Inc group (Novick *et al.*, 1976; Datta and Hughes, 1983). Therefore, plasmids with the same replicon control are deemed 'incompatible' (Novick, 1987).

Due to the number of plasmid Inc types discovered and the time consuming and laborious nature of Inc typing, rapid and less demanding molecular assays were designed to identify replication genes corresponding to the incompatibility type (Carattoli *et al.*, 2006). In 1988, Couturier *et al.* developed a hybridisation method which recognised 19 different replication gene types or 'replicons'. This method paved the way for PCR detection methods using the replication genes as target regions. The first PCR based categorisation method was limited to four Inc types (Gotz *et al.*, 1996); Carattoli *et al.*, (2005) further developed a functional and relatively easy PCR assay to characterise all the major Inc types, revolutionising the way plasmids are grouped (Carattoli *et al.*, 2005; Carattoli *et al.*, 2006).

Despite the universal acceptance of the PCR method, many limitations remain. Sequencing of *rep* genes has shown that a few base-pair changes can alter the incompatibility type of a plasmid; therefore mutations in the PCR primer binding sites could change the Inc status, whilst the plasmid backbone remains unchanged.

Additionally, *rep* genes may be under considerable selective pressure within bacterial cells if interacting with other plasmids. Multiple types of *rep* gene have also been identified on single plasmid backbones allowing the plasmid membership of more than one group (Osborn *et al.*, 2000). Due to the dynamic nature of this genetic region, determining the incompatibility type alone is limited in its ability to inform on the plasmid evolution and the plasmid type as a whole (Francia *et al.*, 2004).

1.4.1.2 *MOB relaxase typing*

In order to overcome some of the limitations of *rep* typing, an alternative plasmid typing method has been developed, although it is not used widely. ‘MOB’ typing is based on the genotype of the relaxase gene, believed to be the most universally common feature of both conjugative and mobilisable plasmids (Francia *et al.*, 2004). The relaxase protein initiates and terminates conjugation by catalysing cleavage of the DNA during transfer to a recipient cell. Therefore, its activity is essential for this process to occur and a relaxase gene is present in all conjugative plasmids (Smillie *et al.*, 2010). PCR primers for each of six groups are used to amplify regions of the relaxase for identification. Further analysis can be achieved by sequencing these amplimers, providing information on phylogeny and further discrimination between plasmids (Garcillan-Barcia *et al.*, 2009).

1.4.1.3 *Plasmid multi-locus sequence typing (pMLST)*

Other methods of plasmid characterisation include the adoption of principles used for multi-locus sequence typing (MLST). By amplifying and sequencing selected ‘housekeeping’ genes a sequence type can be generated for further categorisation.

Plasmid MLST (PMLST) has been applied to both IncI1 and IncHI1 plasmids with some success (Garcia-Fernandez *et al.*, 2008; Phan *et al.*, 2009). However, this method is not suitable for plasmid groups with either great diversity or homology due to the limited number of loci examined and the mosaic composition of distantly related plasmids (Villa *et al.*, 2010).

1.4.2 Incompatibility group I complex plasmids

Currently, plasmids from twenty-six different Inc groups have been identified amongst *Enterobacteriaceae* species (Johnson *et al.*, 2007). However, it is the IncI complex which has the most relevance to this study. Members of the IncI complex have narrow host ranges (Suzuki *et al.*, 2010) and have replication systems which rely on inhibition of pseudoknot formation, providing antisense RNA-mediated inhibition including incompatibility groups I1, I2, Iy; B, O and K (Asano *et al.*, 1999). Other determining characteristics include the presence of two conjugation systems (*tra* and *pil* loci) encoding a 'thick' and 'thin' pilus and a multiple inversion system known as a shufflon, which facilitates variation of the thin pilus tip protein, determining specificity of host binding (Komano *et al.*, 1995; Sampei *et al.*, 2010). Despite their ubiquitous nature, little is known about the IncI complex group of plasmids and even less has been documented regarding groups B, O and K, which are thought to be most closely related to one another.

1.4.2.1 *Complete DNA sequences of IncI complex plasmids*

The genomes of over 800 plasmids from the γ -Proteobacteria have been fully sequenced (Carattoli, 2009) creating a wealth of information with which to compare genotypic attributes of different plasmids. The first IncI1 plasmids to be sequenced included R64 (AP005147) (Meynell and Datta, 1966; Sampei *et al.*, 2010) isolated

from an *S. Typhimurium* strain and Collb-P9 from a *Shigella sonnei* isolate (AB021078) (Mankovich *et al.*, 1986). Both plasmids are often used as reference sequences for this group due to their comprehensive annotations. Other plasmids used for reference include those sequenced by the Sanger Institute (<http://www.sanger.ac.uk/Projects/Plasmids/>), for example IncB plasmid TP113 (NCTC 50084) and IncK plasmid R387 (NCTC 50022). Many other IncI complex plasmid sequences are available (<http://www.ncbi.nlm.nih.gov/nucleotide/>) ranging from plasmids which have been isolated from pathogenic bacteria and/or carry antibiotic resistance genes, to those isolated from the environment; although few have been rigorously annotated.

1.4.3 Epidemiology of plasmids carrying antibiotic resistance genes

The significance of the ability of plasmids to mobilise antibiotic resistance genes between bacteria was first acknowledged in the 1960's in Watanabe's work which identified the need for a replication and transfer system in addition to the movable antibiotic resistance genes on plasmids (Watanabe, 1963). This was followed by the discovery of plasmid mediated β -lactamase genes (Bradford, 2001) and by the demonstration, using early molecular techniques, that the same plasmid could be identified in different bacterial strains and species during an outbreak of *Klebsiella pneumoniae* (Sadowski *et al.*, 1979). Since this time there has been an explosion in the frequency and diversity of β -lactamase genes found on plasmids and an increase in their clinical significance. Over 1000 resistance plasmids have now been typed into the 27 currently known incompatibility groups. However, despite the growing importance of this field, very little is known about the distribution, evolution and selective pressure of antimicrobial resistance carrying plasmids.

1.4.4 Plasmid evolution and evolutionary fitness

Evolutionary fitness can be defined as the capability of an 'evolutionary unit' to survive and reproduce within a given environment (Andersson and Hughes, 2010). Therefore, success of an individual plasmid within a bacterial population is dictated by many factors. These include the presence or absence of selective pressures, the stability of the plasmid within the bacterium and any fitness cost imposed on the host by the plasmid's residence. Despite the multi-factorial nature, bacterial and plasmid fitness is commonly assessed simply using differences in bacterial growth rates either in pure culture or in paired competition (Pope *et al.*, 2010). For many years it was believed that all plasmids, particularly those carrying antibiotic resistance genes conferred a fitness burden on the bacterial host. So much so that in the absence of antibiotic pressure these plasmids would be outcompeted by their plasmid free counterparts reversing the dominance of resistance phenotypes (Andersson and Levin, 1999). Supporting evidence came from the many examples of chromosomal antibiotic resistance determinants which had negative fitness effects on their hosts (Björkman and Andersson, 2000), and studies examining the burden early molecular vectors initially had on laboratory strains of bacteria. Some *in vivo* studies also suggested that a selective pressure was required for plasmids to persist within the human intestinal tract (Anderson, 1974).

In the majority of cases the presence of a plasmid within a naive bacterial host has resulted in some fitness cost (Lenski, 1998); this may be related to the metabolic cost of replication and maintenance of the plasmid (Dionisio *et al.*, 2005), due to plasmid encoded protein expression, or may be caused by disruption of cellular regulation (Rozkov *et al.*, 2004). However, experiments with plasmids pCYC184 and pBR322

have shown that a period of co-evolution of host and plasmid may alleviate these metabolic burdens (Lenski *et al.*, 1994). There are examples in the literature where host chromosomal changes have facilitated this amelioration (Bouma and Lenski, 1988); where mutation of both host and plasmid genes has been observed (Dahlberg and Chao, 2003); and examples of co-evolution yielding an evolutionary fitter individual when competed with the plasmid free parent (Enne *et al.*, 2004).

Studies in humans and animals have also shown that both antibiotic resistant bacterial strains and plasmids can be stably maintained within the gut in the absence of any antibiotic pressure (Smith, 1975; Petrocheilou *et al.*, 1976; Chaslus-Dancla *et al.*, 1987), and that amelioration and selection of more competitive strains can also occur *in vivo* in the human and poultry intestinal tract (Bjorkholm *et al.*, 2001; Luo *et al.*, 2005). Antibiotic resistance genes carried on plasmids are usually constitutively expressed. This means that any fitness burden or benefit conferred by the resistance gene will be indistinguishable from effects of the carrier plasmid upon the host, especially if the plasmid copy number per cell is greater than that of the host chromosome. Examples of inducible plasmid borne resistance genes have also been identified; the fitness burden is reduced in the absence of antibiotic as production of the enzyme is 'switched off', for example inducible vancomycin resistance in *S. aureus* (Foucault *et al.*, 2009). Other studies also demonstrate that it may not be enzyme production alone that is responsible for the fitness burden of these plasmid borne genes, and that signal sequences or transcription regulators may also have a role to play (Macvanin *et al.*, 2004; Marciano *et al.*, 2007).

The cost of one antibiotic resistance gene may also be obscured by the presence of another on the same plasmid. An environment which favours selection of one

antibiotic resistance gene determinant will allow evolutionary success for all genes encoded on the plasmid. Co-selection is thought to have facilitated the spread of many plasmid-borne genes and has been demonstrated to aid the persistence of plasmids (Enne *et al.*, 2001; Bean *et al.*, 2009). It may be that co-selection has also driven the evolution of large multi-drug resistance plasmids now commonly identified (Woodford *et al.*, 2009; Jiang *et al.*, 2010). Although the parameters of plasmid fitness and success are largely unknown, plasmid encoded genes that play a role in promotion of successful persistence include those encoding post segregational killing systems, active partitioning systems (Summers, 1998; Hayes, 2003; Enne *et al.*, 2005) and the strict regulation of copy number (Dahlberg and Chao, 2003; Loftie-Eaton and Rawlings, 2010).

Transfer by conjugation is also considered a key factor in plasmid success both within and between bacterial populations (Bahl *et al.*, 2007). However, the absence of an effective way to measure the effect of conjugation on population dynamics has hindered investigations (Haft *et al.*, 2009). Stewart and Levin (1977) outlined the first comprehensive mathematical model of bacterial fitness based on a mass action principle which took into account numerous variables such as growth rates, transfer rates and plasmid loss (Stewart and Levin, 1977). However, the model does not include differences in bacterial growth phases, depression of pili formation e.g. after conjugation has just occurred, and does not account for environmental factors such as stress, liquid vs. solid surface mating and the presence of biofilms (Sorensen *et al.*, 2005; Slater *et al.*, 2008). It is widely believed that conjugation will occur at much higher frequencies in the environment than seen in the laboratory as the limited

studies performed *in vivo* have shown higher rates of plasmid transfer (Sorensen *et al.*, 2005; Hunter *et al.*, 2008).

1.4.5 Plasmid control measures

Currently, there are no therapeutic measures for plasmid control. This is likely to be due to a number of factors, i) the heterogenic nature of plasmids means a 'broad spectrum' anti-plasmid molecule with action against all incompatibility types is unlikely to exist; ii) the presence of plasmid encoded post segregation killing mechanisms actively select against plasmid free cells; iii) many of the successful curing agents are toxic to humans and animals such as ethidium bromide or SDS, making them unsuitable for treatments; and iv) a lack of effective, high-throughput methods to assess loss of a plasmid both *in vitro* and *in vivo* (Amábile-Cuevas and Heinemann, 2004). However, there are three anti-plasmid strategies detailed within the literature which exploit the basic plasmid functions; inhibition of plasmid replication, use of the toxin in post segregational killing systems (PSK), and the inhibition of plasmid conjugation (Williams and Hergenrother, 2008).

The inhibition of plasmid replication already exists in nature in the form of plasmid incompatibility where one plasmid will prevent the stable inheritance of another. Studies have shown that small molecules (such as apramycin) can mimic incompatibility by binding to the mRNA which controls replication in IncB plasmids, therefore preventing replication and eliminating the plasmid from a bacterial population (DeNap *et al.*, 2004; Thomas *et al.*, 2005). Hale *et al.*, (2010) successfully 'cured' plasmid p0157 using a competing plasmid (pCURE) which encoded a anti-replication molecule and an 'antidote' to neutralise toxins produced by the p0157 PSK regions (Hale *et al.*, 2010). Although very effective, this bespoke method is

currently plasmid specific and requires knowledge of replication and PSK mechanism encoded, therefore, is at present not suitable for anti-plasmid treatment.

The second strategy simply prevents transcription, translation or binding of the anti-toxin component of toxin-antitoxin (or PSK) systems, therefore freeing the toxin and causing cell death (Williams and Hergenrother, 2008). This method has the advantage that the bacterial cell is killed in addition to the removal of the plasmid from the population, and as PSK systems are found in most large conjugative plasmids, targeting the common PSK antitoxins may have a broad spectrum of action. Few compounds with antitoxin action have been identified so far, therefore the viability of this method is currently unknown.

The prevention of plasmid conjugation is the third strategy proposed which although does not remove the plasmid from a population or kill bacterial cells, could be used as a prophylactic measure to prevent plasmid spread within particular environments, e.g. the gastrointestinal tract of humans or livestock animals. Essential conjugation initiation and termination proteins (relaxases) have been identified as a potential target for inhibition as they are found in all conjugative plasmids. Clinically and veterinary approved drugs, the bisphosphonates and flavomycins, have been shown to inhibit the action of these proteins *in-vitro* and bisphosphonates had some action *in vivo*, suggesting this may be a promising treatment option (Poole *et al.*, 2006; Lujan *et al.*, 2007). An alternative method for preventing conjugation is the use of bacteriophage therapy. Phage (for example M13) are able to bind to the conjugation apparatus either preventing the initiation of donor and recipient binding (Novotny *et al.*, 1968; Jalasvuori *et al.*, 2011) or causing the pilus to retract back into the cell (Jacobson, 1972) therefore preventing successful conjugation. Lin *et al.*, (2011)

showed that phage coat proteins are able to attach to the tip of conjugation pilus and prevent conjugation without the presence of a replicating filamentous phage (Lin *et al.*, 2011), therefore the administration of phage proteins may also be a tenable therapeutic anti-conjugation option in the future.

1.4.6 Plasmid mediated *bla*_{CTX-M-14}

Nearly all *bla*_{CTX-M} genes outside the *Kluyvera spp.* are located on plasmids, and variants *bla*_{CTX-M-9}, *bla*_{CTX-M-14}, *bla*_{CTX-M-15} and *bla*_{CTX-M-32} are associated with 'epidemic' plasmids found in a number of geographical locations (Cantón and Coque, 2006). Associations have been made between *bla*_{CTX-M-15} and IncF epidemic plasmids, particularly IncFII (Novais *et al.*, 2007). In contrast, current reports indicate that *bla*_{CTX-M-14} can be found on a wide range of different plasmid types including Inc groups A/C; FIB and FII, I1, P, L/M and K (Pai *et al.*, 2001; Hopkins *et al.*, 2006; Bae *et al.*, 2008; Marcade *et al.*, 2008; Kang *et al.*, 2009). These plasmids range in size from 35 kb (pBDE0502, from a clinical *E. coli* isolate from Korea) to 160 kb (pMG267, from a *Klebsiella pneumoniae* also from Korea) and are found within many different bacterial species (Bae *et al.*, 2008; Pai *et al.*, 2001). At the start of this project no *bla*_{CTX-M-14} carrying plasmids had been sequenced, but as of September 2011, three *bla*_{CTX-M-14} carrying plasmids have been deposited and annotated in Genbank. The sequence of plasmid pCT is described in this project (FN868832)(Cottell *et al.*, 2011), pTN48, an IncFII/FIB plasmid was from an *E. coli* isolated in Paris (165,692 bp- FQ482074)(Billard-Pomares *et al.*, 2011) and pKF3-70 was identified in a *K. pneumoniae* from China (70,057 bp - FJ4949913) (Yi *et al.*, 2010).

1.4.6.1 *bla*_{CTX-M-14} on *IncK* plasmids

Plasmids of the incompatibility group *IncK* have been identified in bacteria isolated from medical isolates, particularly in urinary tract infections since the early 1970s (Tschape and Tietze, 1980; Valverde *et al.*, 2009). However, it is only recently that this under studied group has been associated with the dissemination of antibiotic resistance genes. *IncK* plasmids carrying *bla*_{CTX-M-14} are most commonly found in Spain where *bla*_{CTX-M-14} is the most frequently isolated ESBL. *IncK* plasmids carrying *bla*_{CTX-M-14} have been isolated from *E. coli* and *Salmonella* (Gonzalez-Sanz *et al.*, 2009) from animals (Blanc *et al.*, 2006; Blanc *et al.*, 2008), collected from patients and healthy humans, and found in environmental samples (Navarro *et al.*, 2007; Diestra *et al.*, 2008). To date, *bla*_{CTX-M-14} carrying *IncK* plasmids have also been documented in France and Australia, isolated from patient *E. coli* strains (Marcade *et al.*, 2008; Zong *et al.*, 2008) as well as in cattle in the UK (Teale *et al.*, 2005). Due to the widespread distribution of these bacteria, many authors have referred to the spread of *bla*_{CTX-M-14} on *IncK* plasmids as a plasmid ‘epidemic’, particularly in Europe (Baudry *et al.*, 2009; Carattoli, 2009). It has been proposed that it is the same *IncK* plasmid that has disseminated throughout these environments. A recent study in Spanish hospitals identified and examined *bla*_{CTX-M-14} carrying plasmids from *E. coli* clinical samples (Valverde *et al.*, 2009). Plasmid pRYC105 was the predominant plasmid found, identified in bacteria from patients, outpatients and from healthy volunteers from 2000-2005. This plasmid was identified in a number of different *E. coli* strains so its dissemination cannot be attributed to clonal expansion, therefore it is likely that it is the plasmid itself which has disseminated into these different environments.

1.5 Background to this study, *E. coli* isolate C159/11 and *bla*_{CTX-M-14} carrying IncK plasmid pCT

In 2004 Teale *et al.*, (2004) isolated an *E. coli* from diarrhoeic calves on a dairy farm in Wales containing a *bla*_{CTX-M-14} β -lactamase on a 65 MDa plasmid. This *E. coli* had atypical biochemical characteristics such as a negative indole result and did not produce common *E. coli* toxins (Teale *et al.*, 2005). C159/11 was resistant to ampicillin, 1st-4th generation cephalosporins, chloramphenicol, streptomycin, sulfamethoxazole, trimethoprim, tetracycline, sulfonamide, nalidixic acid and ciprofloxacin (Liebana *et al.*, 2006). This isolate also contained another β -lactamase *bla*_{TEM-35} (IRT-4) gene, encoded on a second, smaller plasmid giving additional resistance to amoxicillin/clavulante combinations (Speldooren *et al.*, 1998). Although the *E. coli* C159/11 was not the primary cause of the pathology and subsequent high mortality in the calves, a longitudinal study from December 2004 to July 2005 was initiated by the Veterinary Laboratories Agency to monitor the dissemination of this *bla*_{CTX-M-14} gene. Faecal samples and rectal swabs were taken from the then current calf population and from the milking cows on all three sampling occasions, swabs of the floor of the calf house and collecting yard and slurry testing was also carried out on the second and third visits (Teale *et al.*, 2005). On the first sampling occasion 64.6% of calf samples and 3.3% of milking cow samples contained a CTX-M-14 producing isolate. On the second visit 61.8% of calves and 5.9% of cow samples were positive; and on the third occasion 92.7% of calf samples and 23.8% of milking cow samples tested positive for *bla*_{CTX-M-14}. On the third visit the environmental samples also showed *bla*_{CTX-M-14} carrying *E. coli* in slurry and on the floor of surrounding areas. Over the duration of the study PFGE was used to identify six different *E. coli* clones containing the *bla*_{CTX-M-14} carrying plasmid, suggesting

dissemination was not due to clonal expansion of a single bacterial strain alone (Carattoli, 2008). Examination of the plasmids carrying *bla*_{CTX-M-14} from these isolates showed that they were indistinguishable by RFLP typing and classified in the incompatibility group IncK using PCR based replicon typing (Carattoli *et al.*, 2005). Cephalosporins were not administered on the farm for the duration of the study, however, a variety of antimicrobials had been previously used including the fourth generation cephalosporin cefquinome (Teale *et al.*, 2005). The original *E. coli* isolated, C159/11, is believed to be the first reported ESBL producing *E. coli* isolated from livestock in the UK. The plasmid isolated from C159/11 was characterised as being 65 MDa, or ~80 kb in size (Liebana *et al.*, 2006). Subsequent conjugation experiments with this plasmid indicated that horizontal transfer is extremely efficient, which may have led to its dissemination to many different strains. In this project the plasmid isolated from *E. coli* C159/11 was called pCT.

1.6 Hypotheses

- 1) Successful persistence and dissemination of the C159/11 plasmid pCT within a population is related to an evolutionary fitness advantage transferred to the host bacterium by this plasmid.
- 2) pCT confers altered fitness to host strains in competition.
- 3) The *bla*_{CTX-M-14} gene plays an important role in the evolutionary success of pCT.
- 4) Other genomic aspects of pCT will also play a role in evolutionary fitness of this plasmid.
- 5) pCT-like plasmids with similar genomic attributes to pCT can be found hosted in bacteria isolated from humans, animals and the environment in a range of geographical regions.

1.7 Aims

- 1) To inactivate *bla*_{CTX-M-14} on plasmid pCT.
- 2) To develop a bioassay to allow the presence of pCT to be identified in various models.
- 3) Whole genome sequencing of pCT.
- 4) To compare the pCT sequence with other sequenced plasmids including those of similar incompatibility groups and plasmids carrying *bla*_{CTX-M} genes.
- 5) To determinate the prevalence of pCT-like plasmids using a multiplex PCR assay in bacteria isolated from humans, animals and the environment in a range of geographical locations.
- 6) To investigate the fitness contributions of *bla*_{CTX-M-14} and other pCT genes.
- 7) To assess fitness costs or benefits conferred by pCT *in vitro* and *in vivo* models.

Chapter 2:

Materials and Methods

2. Materials and Methods

2.1 Bacterial strains and growth conditions

The *bla*_{CTX-M-14} carrying IncK plasmid investigated in my study was isolated from an *E. coli* isolate C159/11, obtained by the Veterinary Laboratory agency (VLA) from a cattle farm. This plasmid was named 'pCT' and was studied in several bacterial hosts including *Escherichia coli* and *Salmonella enterica* serovar Typhimurium strains (Table 2.1). All bacterial strains used in this study (Tables 2.1, 2.2, 2.3 and 2.4) were routinely grown on Luria-Bertani (LB) agar (Sigma-Aldrich Ltd., UK, Cat. no. L2897) supplemented with appropriate antibiotics as required, and incubated aerobically overnight at 37°C unless otherwise stated. Overnight broth cultures were grown in 10 ml LB broth (Sigma-Aldrich Ltd., UK, Cat. no. L3522) supplemented with appropriate antibiotics where necessary, and typically incubated aerobically overnight at 37°C with shaking at 225 rpm. When growing strains containing plasmid pCT (or other plasmids encoding *bla*_{CTX-M-14}), both on agar and in broth, the medium was supplemented with 8 µg/ml of cefotaxime (Sigma-Aldrich, UK, Cat. no. C7912) to ensure plasmid maintenance. When growing strains containing mutated pCT the media was supplemented with 50 µg/ml of kanamycin (pCT2-8) (Sigma-Aldrich, UK, Cat. no. K4000) or 20 µg/ml of chloramphenicol (pCT9) (Sigma-Aldrich, UK Cat. no. C0378). Fresh stock solutions of these antibiotics were made on the day of use. ProtectTM beads (Technical Service Consultants Ltd., UK, Cat. no. TS70) were used for long term storage of strains. *E. coli* strains were stored at -80°C and *Salmonella* strains at -20°C. All isolates were initially grown on xylose lysine deoxycholate (XLD) selective agar (Sigma-Aldrich Ltd., UK, Cat. no. 95586) overnight at 37°C. On XLD media, *Salmonella spp.* appear pink with black centres indicating the production of

Table 2.1. Bacterial host strains +/- wild-type pCT

| Laboratory Code | Species/serovar | Strain | Description/ Phenotype | Source/ Reference |
|-----------------|-----------------------|-----------------------------|---|--|
| I753 | <i>E. coli</i> | C159/11 | Veterinary isolate from the AHVLA (pCT) | Teale <i>et al.</i> 2004 Liebana <i>et al.</i> 2006 |
| I825 | <i>E. coli</i> | DH5α | Genotype: F ⁻ , <i>deoR</i> , <i>endA1</i> , <i>recA1</i> , <i>relA1</i> , <i>gyrA96</i> , <i>hsdR17</i> (rk ⁻ , mk ⁺), <i>supE44</i> , <i>thi-1</i> , <i>phoAΔ</i> , (<i>lacZYA argF</i>), U169 Φ80 <i>lacZΔM15</i> λ ⁻ | Invitrogen, UK, Cat. no. 18290-015 |
| I847 | <i>E. coli</i> | J53-2 | NCTC 50167, K12 pro ⁻ and meth ⁻ (<i>lac</i> ⁺) <i>rpoB</i> , F ⁻ (Acridine orange used to cure F ⁺ factor) | Clowes and Rowley, 1954 |
| I823 | <i>E. coli</i> | 3950 | C159/11 derived pCT 'cured' strain | AHVLA* |
| L354 | <i>S. Typhimurium</i> | SL1344 | <i>Salmonella enterica</i> serovar Typhimurium Pathogenic veterinary strain from cattle | Wray and Sojka, 1978 |
| L1078 | <i>S. Typhimurium</i> | SL1344 rif ^R | L354 rifampicin resistant derivative | This study |
| I755 | <i>E. coli</i> | DH5α pCT | I825 pCT transformant | This study |
| I855 | <i>E. coli</i> | J53-2 pCT | I847 pCT transconjugant | This study |
| I834 | <i>E. coli</i> | 3950 pCT | I823 pCT transconjugant | This study |
| L1079 | <i>S. Typhimurium</i> | SL1344 rif ^R pCT | L1078 pCT transconjugant | This study |

* Animal Health and Veterinary Laboratories Agency

Table 2.2. Other bacteria strains used in this study

| Lab code | Species/serovar | Strain | Description/ Phenotype | Source/ Reference |
|----------|-----------------------|------------------------------|---|---|
| I597 | <i>E. coli</i> | DH5 | NCTC 13400 | NCTC |
| I754 | <i>E. coli</i> | DH5 rif ^R | NCTC 13400 rifampicin resistant | This study |
| I759 | <i>E. coli</i> | SW102 | Chromosomal λ -red recombinase strain | Sharan <i>et al.</i> 2009 |
| I879 | <i>E. coli</i> | SW102 pCT | pCT SW102 transformant | This study |
| L1019 | <i>S. Typhimurium</i> | SL1344 pUA66 <i>pacpP</i> | SL1344 with pUA66 <i>pacpP</i> , <i>aph-gfpmut2</i> (kanamycin resistant and constitutively expresses a stable GFP) | Zaslaver <i>et al.</i> , 2006 |
| I355 | <i>E. coli</i> | DH5 α pUC19 | Ampicillin resistant control plasmid for transformations | Invitrogen, Cat. no. 18290-015 |
| I113 | <i>E. coli</i> | | NCTC 10418 | NCTC |
| I633 | <i>E. coli</i> | DH5 α pKD4 | Plasmid encoding an <i>aph</i> kanamycin resistance cassette | Datsenko and Wanner, 2006 |
| L109 | <i>S. Typhimurium</i> | SL1344 <i>tolC::aph</i> | L354 containing a chromosomal <i>aph</i> kanamycin resistance cassette | Buckley <i>et al.</i> , 2006 |
| L829 | <i>S. Typhimurium</i> | L828 <i>tolC::cat</i> | L828 containing a <i>cat</i> chloramphenicol resistance cassette | Nishino <i>et al.</i> , 2006 |
| L828 | <i>S. Typhimurium</i> | 14028s | ATCC 14028S, positive control for biofilm formation | ATCC |
| I845 | <i>E. coli</i> | 042 | Positive control for aggregation | Kindly provided by Prof. Ian Henderson, U of B |

Table 2.3. *E. coli* isolates investigated for the presence of pCT-like plasmids

| Origin | Date | Location | Lab no. | Strain/ plasmid | Inc type | Source |
|--------|---------|---------------|---------|--------------------|----------|------------------------------|
| Cattle | 2004 | England/Wales | I753 | C159/11 pCT | K | Liebana <i>et al.</i> 2006 |
| Cattle | 2006 | England/Wales | I779 | I779 | F,K | AHVLA UK |
| Cattle | 2008 | England/Wales | I780 | I780 | F,K | AHVLA UK |
| Cattle | 2008 | England/Wales | I781 | I781 | FIA | AHVLA UK |
| Cattle | 2009 | England/Wales | I782 | I782 | F | AHVLA UK |
| Cattle | 2007 | England/Wales | I783 | I783 | Unknown | AHVLA UK |
| Cattle | 2008 | England/Wales | I784 | I784 | Unknown | AHVLA UK |
| Cattle | 2008 | England/Wales | I785 | I785 | Unknown | AHVLA UK |
| Cattle | 2006 | England/Wales | I786 | I786 | I1-γ | AHVLA UK |
| Cattle | 2006 | England/Wales | I787 | I787 | Unknown | AHVLA UK |
| Cattle | 2008 | England/Wales | I788 | I788 | Unknown | AHVLA UK |
| Cattle | 2008 | England/Wales | I789 | I789 | Unknown | AHVLA UK |
| Cattle | 2006 | England/Wales | I790 | I790 | Unknown | AHVLA UK |
| Cattle | 2008 | England/Wales | I791 | I791 | F | AHVLA UK |
| Cattle | 2008 | England/Wales | I792 | I792 | F | AHVLA UK |
| Cattle | 2008 | England/Wales | I793 | I793 | F | AHVLA UK |
| Human | no data | England/Wales | I804 | L125 | Unknown | P. Hawkey |
| Human | 2006 | Germany | I794 | 386 | FII | Cullik <i>et al.</i> 2010 |
| Human | 2006 | Germany | I795 | 400 | FII | Cullik <i>et al.</i> 2010 |
| Human | 2003-4 | Spain | I796 | C574 | K | Vinue <i>et al.</i> 2008 |
| Human | 2003-4 | Spain | I797 | C559 | K | Vinue <i>et al.</i> 2008 |
| Human | 2003-4 | Spain | I798 | C567 | K | Vinue <i>et al.</i> 2008 |
| Human | 2001-5 | Spain | I809 | FEC383/ pRYC105 | K | Valverde <i>et al.</i> 2009 |
| Human | 2002 | Spain | I810 | E36/ pRYC110 | HI2 | Valverde <i>et al.</i> 2009 |
| Human | 1998 | China | I811 | CH13/ pOZ174 | Unknown | Chanawong <i>et al.</i> 2002 |
| Human | 2005-7 | Australia | I813 | JIE 052 | B | Zong <i>et al.</i> 2008 |
| Human | 2005-7 | Australia | I814 | JIE 081 | FII | Zong <i>et al.</i> 2008 |
| Human | 2005-7 | Australia | I815 | JIE 084 | FII | Zong <i>et al.</i> 2008 |
| Human | 2005-7 | Australia | I816 | JIE 088 | I1 | Zong <i>et al.</i> 2008 |
| Human | 2005-7 | Australia | I817 | JIE 182 | B | Zong <i>et al.</i> 2008 |
| Human | 2005-7 | Australia | I818 | JIE 201 | K | Zong <i>et al.</i> 2008 |

Laboratory numbers of the *E. coli* DH5α pCT-like plasmids transformants can be found in Appendix 2.

Table 2.4. Bacterial strains containing mutant pCT plasmids (pCT2-9)

| Species/ serovar | Host strain | Plasmid | Resistance phenotype | Lab code |
|--|------------------------------------|---|---|-------------|
| <i>E. coli</i> | DH5α (I825) | pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | kan ^R | I877 |
| | | pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | kan ^R | I826 |
| | | pCT3 (Sigma factor:: <i>aph</i>) | CTX ^R + kan ^R | I827 |
| | | pCT4 (<i>rci</i> :: <i>aph</i>) | CTX ^R + kan ^R | I828 |
| | | pCT5 (<i>pilS</i> :: <i>aph</i>) | CTX ^R + kan ^R | I829 |
| | | pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | CTX ^R + kan ^R | I830 |
| | | pCT7 (<i>traXY</i> :: <i>aph</i>) | CTX ^R + kan ^R | I846 |
| | | pCT8 (<i>pndACB</i> :: <i>aph</i>) | CTX ^R + kan ^R | I877 |
| | | pCT9 (<i>traXY</i> :: <i>aph</i> ; <i>pndACB</i> :: <i>cat</i>) | CTX ^R , kan ^R + chl ^R | I878 |
| | J53-2 (I847) | pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | kan ^R | I856 |
| | | pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | kan ^R | I857 |
| | | pCT3 (Sigma factor:: <i>aph</i>) | CTX ^R + kan ^R | I858 |
| | | pCT4 (<i>rci</i> :: <i>aph</i>) | CTX ^R + kan ^R | I859 |
| | | pCT5 (<i>pilS</i> :: <i>aph</i>) | CTX ^R + kan ^R | I860 |
| | | pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | CTX ^R + kan ^R | I861 |
| | | pCT8 (<i>pndACB</i> :: <i>aph</i>) | CTX ^R + kan ^R | I893 |
| | 3950 (I823) | pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | kan ^R | I835 |
| | | pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | kan ^R | I836 |
| | | pCT3 (Sigma factor:: <i>aph</i>) | CTX ^R + kan ^R | I837 |
| | | pCT4 (<i>rci</i> :: <i>aph</i>) | CTX ^R + kan ^R | I831 |
| | | pCT5 (<i>pilS</i> :: <i>aph</i>) | CTX ^R + kan ^R | I841 |
| | | pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | CTX ^R + kan ^R | I838 |
| | | pCT8 (<i>pndACB</i> :: <i>aph</i>) | CTX ^R + kan ^R | I894 |
| | SW102(I759) | pCT7 (<i>traXY</i> :: <i>aph</i>) | CTX ^R + kan ^R | I880 |
| <i>Salmonella</i> <i>enterica</i> serovar Typhimurium | SL1344 rif ^R (L1078) | pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | kan ^R | L1244 |
| | | pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | kan ^R | L1235 |
| | | pCT3 (Sigma factor:: <i>aph</i>) | CTX ^R + kan ^R | L1236 |
| | | pCT4 (<i>rci</i> :: <i>aph</i>) | CTX ^R + kan ^R | L1259 |
| | | pCT5 (<i>pilS</i> :: <i>aph</i>) | CTX ^R + kan ^R | L1260 |
| | | pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | CTX ^R + kan ^R | L1245 |
| | | pCT8 (<i>pndACB</i> :: <i>aph</i>) | CTX ^R + kan ^R | L1397 |

CTX, cefotaxime; kan, kanamycin; chl, chloramphenicol.

hydrogen sulphide (H₂S). *E. coli* ferments lactose and sucrose within the media, changing the pH and resulting in a yellow colour change in the agar. Gram stain and microscopic examination (at 100 x magnification with an oil immersion lens) was also used to confirm cell morphology and cell wall type of each strain. Gram stain reagents were purchased together in a kit (Fisher Scientific, Loughborough, Cat. no. P/L505/15).

2.2 Determination of susceptibility to antibiotics

The agar doubling-dilution method as described by the British Society for Antimicrobial Chemotherapy (BSAC) (Andrews, 2011) was used to determine the minimum inhibitory concentration (MIC) of a range of antibiotics (Table 2.5). Appropriate volumes of the antimicrobial stock were dispensed into universal containers to which 20 ml of molten Iso-sensitest agar (Oxoid, UK, Cat. no. CM0471) cooled to approx 50°C was aseptically added. The antibiotic and agar were mixed and poured into sterile tri-vented Petri dishes and allowed to set. Plates were dried for 20 minutes in a 50°C oven before inoculation. Overnight cultures of each bacterial strain to be tested were diluted 1:100 to give approximately 10⁷ CFU/ml. Each agar plate was inoculated with 1 µl of the diluted culture giving approximately 10⁴ CFU/spot using a 100 pin multipoint inoculator (AQS manufacturing, UK). On each occasion *E. coli* NCTC 10418 (I113) was used as a control strain as recommended by BSAC. Inoculated plates were incubated overnight at 37°C. The MIC was defined as the lowest concentration of the antimicrobial that inhibited 90% of visible growth of the organism after overnight incubation and was determined as the modal value from at least three independent experiments.

Table 2.5. Antimicrobials used in this study

| Agent | Antimicrobial class | Solubilised in | Supplier | Catalogue number |
|-----------------|---|------------------------------|------------------|------------------|
| Ampicillin | Penicillin β lactam | Sodium bicarbonate and water | Sigma-Aldrich UK | A5918 |
| Cefotaxime | 3 rd generation cephalosporin β lactam | Distilled water | Sigma-Aldrich UK | C7912 |
| Ceftiofur | 3 rd generation cephalosporin β lactam | Distilled water | Sigma-Aldrich UK | 34001 |
| Chloramphenicol | Phenicol | 70% methanol | Sigma-Aldrich UK | C0378 |
| Ciprofloxacin | Quinolone | Acetic acid and water | Sigma-Aldrich UK | 17850 |
| Erythromycin | Macrolide | 70% ethanol | Sigma-Aldrich UK | E5389 |
| Faropenem | Carbapenem β -lactam | Distilled water | Sigma-Aldrich UK | F8182 |
| Gentamicin | Aminoglycoside | Distilled water | Sigma-Aldrich UK | G1264 |
| Kanamycin | Aminoglycoside | Distilled water | Sigma-Aldrich UK | K1876 |
| Rifampicin | Rifamycin | Methanol | Sigma-Aldrich UK | R3501 |
| Tetracycline | Tetracycline | Distilled water | Sigma-Aldrich UK | T3258 |

2.3 Plasmid DNA isolation

2.3.1 Mini plasmid preparation

Plasmid DNA from a range of isolates was routinely harvested using the QIAgen® Miniprep kit (QIAgen, UK, Cat. no. 27104) following the low copy number protocol to ensure maximum yield, as described by the manufacturer's instructions. Plasmid DNA was stored at -20°C as aliquots of 10 µl.

2.3.2 Maxi plasmid preparation

The QIAgen® Large construct kit (QIAgen, UK, Cat. no. 12462) was used to extract a high yield of plasmid pCT from strain *E. coli* DH5α pCT (I755) following the manufacturer's instructions. For high speed centrifugation steps volumes of 50-250 ml were placed in 250 ml polypropylene tubes (Beckman Coulter, High Wycombe, Cat. no. 356011) and centrifuged using a Beckman JA25.50 rotor (Beckman Coulter, High Wycombe, Cat. no. 363058). For volumes up to 50 ml polycarbonate tubes (Beckman Coulter, High Wycombe, Cat. no. 357002) were used in a JLA16.250 rotor (Beckman Coulter, High Wycombe, Cat. no. 363930).

2.3.3 Isolation of high quality plasmid DNA for sequencing

To harvest plasmid DNA of sufficient high quality for genome sequencing, an alkaline lysis SDS extraction (Birnboim and Doly, 1979) followed by a caesium chloride gradient clean up (Smith and Thomas, 1983) was used to extract pCT from *E. coli* DH5α pCT (I755).

2.3.3.1 *Birnboim and Doly maxi-preparation*

Cells from 400 ml of overnight LB broth culture supplemented with 8 µg/ml of cefotaxime were harvested using centrifugation for 15 minutes at 2,250 x *g*. The

bacterial cell pellet was re-suspended in 25 ml of ice cold Lysis solution 1 (25 mM Tris pH 8.5, 10 mM EDTA pH 8.0, 50 mM glucose) supplemented with RNase (20 µg/ml) (Ambion, Texas, Cat. no. RM2290) to reduce the RNA yield in the final sample. Fifty microliters of freshly prepared Lysis solution 2 (0.2 M NaOH, 1% SDS) was then added and the sample inverted to ensure complete lysis. After incubation on ice for five minutes, 37.5 ml of Neutralising solution 3 (3 M sodium acetate pH 5.0) was added and solution mixed carefully by inversion, followed by a further five minute incubation on ice. Proteins and cell debris were removed by centrifugation at 10,000 x g at 4°C for 15 minutes. The supernatant was then passed through filter paper to remove any remaining cell debris. To precipitate the plasmid DNA from the filtered supernatant, 100 ml of isopropanol was added followed by centrifugation at 14,000 x g for 15 minutes at room temperature. The supernatant was subsequently discarded and DNA pellet resuspended in 3 ml of 1x TNE buffer (100 mM Tris pH 8.0, 50 mM NaCl, 5 mM EDTA pH 8.0).

2.3.3.2 *Caesium chloride density gradient centrifugation*

The exact volume of suspended plasmid DNA was measured in µl using a pipette, and 4.72 g of caesium chloride (CsCl) added. TNE x 1 buffer (100 mM Tris pH 8.0, 50 mM NaCl, 5 mM EDTA pH 8.0) was then added to make an exact total volume of 4.5 ml. The solution was gently inverted until the CsCl dissolved. Five hundred microlitres of 10 mg/ml ethidium bromide (EtBr) was also added and the solution divided exactly into two 3.5 ml ultra-centrifugation tubes (Beckmann Coulter, High Wycombe, Cat. no. 349621) using a syringe or Pasteur pipette. Weighing ensured both tubes were of equal weight (within 0.1 g of each other) then 1 x TNE buffer was used to fill tubes to the top. The application of heat using a QuickSeal® tube

topper (Beckmann Coulter, High Wycombe, Cat. no. 360976) sealed the tops of the tubes which were centrifuged for 24 hours at 20°C at 801,920 x *g* in a Beckmann Coulter ultracentrifuge with a Type 100 Ti rotor and spacer (Beckmann Coulter, High Wycombe, Cat. no. 360270). After the removal of the heat sealed lids using scissors, the ultracentrifuge tubes were visualised under UV light and plasmid DNA, seen as fluorescent bands, was extracted using a needle and syringe.

Ethidium bromide was removed from the plasmid DNA by adding isopropanol saturated with caesium chloride and water, and shaking vigorously. The separated isopropanol 'top layer' containing the ethidium bromide was then removed and discarded and this step repeated a minimum of three times. To the cleaned sample (400 µl) 400 µl of nuclease free water, 100 µl of 3 M Sodium Acetate and 530 µl of isopropanol was added and centrifuged for 10 minutes at 13,000 x *g* to further clean the plasmid DNA. Once the supernatant had been removed, the plasmid DNA was re-suspended in 1 x TNE buffer and 25 µl of 3 M sodium acetate and 500 µl of ethanol before centrifugation at 12,100 x *g* for 10 minutes. The pellet was washed further using 70% ethanol. Once air dried, plasmid DNA was re-suspended in 50 µl 10 x TNE buffer. The DNA (3µl) was separated using electrophoresis on a 0.7% agarose gel and plasmid DNA quantified using a spectrophotometer.

2.4 Complete plasmid sequence analysis

2.4.1 Sequencing and annotation

The plasmid DNA sequence was determined using a 454/Roche GS FLX 382 analyser and assembled *de novo* using the 454/Roche Newbler assembly program (Chain *et al.*, 2009). Initial annotation at the Sanger Institute used Artemis

(<http://www.sanger.ac.uk/resources/software/artemis/>). Further comparative analysis of the DNA sequence was completed as part of this project at The University of Birmingham. Graphic representation of the sequence in the form of a plasmid map was constructed using DNASTar (Lasergene, Madison).

2.4.2 Comparative genomics

Double ACT (http://www.hpa-bioinfotools.org.uk/pise/double_act.html) and the Artemis Comparison Tool (ACT) (<http://www.webact.org/WebACT/home>) were used to compare the pCT sequence to other plasmid sequences on a whole genome scale. Genogator (<http://www.kato.mvc.mcc.ac.uk/genogator/doc/about.htm>) was used to align particular regions or operons of pCT against the same regions in other plasmids and BLAST ([National Center for Biotechnology Information](http://www.ncbi.nlm.nih.gov/BLAST/)) was used to examine particular coding sequences (CDSs) in more detail, for more comprehensive annotations and for further comparisons against plasmids of known sequence.

2.5 Amplification and DNA sequencing of genomic elements on pCT

PCR was used throughout this study to confirm the presence of plasmid pCT and the antibiotic resistance gene *bla*_{CTX-M-14} during routine checks and for confirmation of pCT transfer into new bacterial host strains. PCR was also used for detection of pCT-like plasmids and to explore the insertion site of *bla*_{CTX-M-14} within each of the pCT-like plasmids identified.

2.5.1 Polymerase chain reaction

Target DNA for PCR was isolated by suspending colonies from test isolates in 50 µl of sterile distilled water and heating at 99°C for 5 minutes, followed by centrifugation at 13,000 rpm in a micro-centrifuge to pellet the cell debris. All PCR assays were

carried out in a 50 µl volume consisting of 45 µl of PCR ReddyMix (containing a Taq polymerase) (ThermoScientific, USA, Cat. no. AB0794), 2 µl of the supernatant taken from boiled cell lysates, 1 µl of sterile distilled water and 1 µl each of forward and reverse primers. Primers were purchased from Invitrogen, UK (Table 2.3 and 2.4), used at a working concentration of 25 µM and stored at -20°C. After initial treatment at 95°C for 5 minutes, typically the PCR was carried out for 30 cycles consisting of denaturation at 95°C for 30 seconds, annealing at 51°C for 30 seconds and elongation at 72°C for 40 seconds and 10 minutes at 72°C. A contamination control containing master mix, primers and water (replacing template DNA) was included in all PCR reactions and results were observed using agarose gel electrophoresis.

2.5.2 Agarose gel electrophoresis

All PCR amplimers were separated by electrophoresis on 1% agarose gels, containing 1 g of electrophoresis grade agarose (Invitrogen Ltd, UK, Cat. no. 15581-028) and 100 ml of 1% Tris-Boric acid-EDTA (TBE, 10 x TBE buffer contains 108 g Tris, 9.3 g EDTA and 55 g Boric acid per litre) (Invitrogen Ltd, UK, Cat. no. 15581-028). Agarose was dissolved by heating and allowed to cool before ethidium bromide was added to a final concentration of 0.1 µg/ml. Typically, 5 µl of sample was added to each well along with a DNA sizing ladder Hyperladder I (Bioline, London, Cat. no. BIO-33025) and electrophoresis was used to separate samples at 100 V for one hour. Amplimers were visualised on a UV transilluminator and where appropriate, DNA quantified using a Gene Genius image analyser (Syngene, Cambridge, UK). Plasmid DNA was visualised using electrophoresis on 0.7% agarose gels (0.7 g of agarose in 100 ml of TBE buffer) at 80 V for 3 hours due to their large size and to separate plasmid DNA with any residue chromosomal DNA.

Table 2.6. Primers used for amplification of specific regions on pCT and detection of pCT-like plasmids

| Lab no. | Primer | Sequence 5' → 3' | Target | Size (bp) | pCT binding site | Reference |
|---------|-----------------|------------------------|---|-----------------------|------------------|--------------------------------|
| 819 | CTX-M-G9 (F) | ATGGTGACAAAGAGAGTGCAAC | <i>bla</i> _{CTX-M} Group 9 variants | 876 | 70259-70280 | Batchelor <i>et al.</i> , 2005 |
| 820 | CTX-M-G9 (R) | TTACAGCCCTTCGGCGATG | <i>bla</i> _{CTX-M} Group 9 variants | 876 | 69405-69423 | Batchelor <i>et al.</i> , 2005 |
| 837 | ISEcp1A (F) | GCAGGTCTTTTTCTGCTCC | Insertion sequence ISEcp1 | 527 | 71728-71746 | Karim <i>et al.</i> , 2001 |
| 838 | ISEcp1B (R) | ATTTCCGGAGCACCGTTTGC | Insertion sequence ISEcp1 | 527/1037 ^a | 71220-71239 | Karim <i>et al.</i> , 2001 |
| 839 | B3A (F) | AACGGCACAATGACGCTGGC | Insertion sequence IS903 | 887 | 69913-69932 | Navarro <i>et al.</i> , 2007 |
| 840 | IS903 (R) | TGTAATCCGGCAGCGTA | Insertion sequence IS903 | 887 | 69045-69061 | Navarro <i>et al.</i> , 2007 |
| 905 | Pseudo (R) | AACATTCGGCCGTTTACAGC | pCT region downstream of <i>bla</i> _{CTX-M-14} | 1636 | 68644-68663 | This study |
| 904 | <i>traK</i> (F) | GGTACCGGCATCGCACAGAA | pCT region upstream of ISEcp1 | 1037 | 72238-72257 | This study |
| 868 | Sigma (F) | ACAGCGTCTTCTCGTATCCA | pCT putative sigma factor | 1289 | 48590-48609 | This study |
| 869 | Sigma (R) | GTTCTTCCAGCTGACGTAAC | pCT putative sigma factor | 1289 | 47320-47339 | This study |
| 875 | <i>rci</i> (F) | AAGGTCATCTGCAGGAGT | pCT shufflon recombinase | 945 | 78364-78381 | This study |
| 876 | <i>rci</i> (R) | GTGTGCGCAGCAACAATA | pCT shufflon recombinase | 945 | 77436-77453 | This study |
| 916 | <i>pilN</i> (F) | GACAGGCAGAGAACACCAGA | pCT <i>pilN</i> outer membrane protein | 627 | 88267-88286 | This study |
| 917 | <i>pilN</i> (R) | ATGCTGTTCCACCTGATGAG | pCT <i>pilN</i> outer membrane protein | 627 | 87659-87678 | This study |
| 979 | <i>nikB</i> (F) | CGTGCMTGCCGTGARCTT | Incl complex <i>nikB</i> relaxase gene | 290 | 33077-33094 | This study |
| 980 | <i>nikB</i> (R) | TCCCAGCCATCCWTCACC | Incl complex <i>nikB</i> relaxase gene | 290 | 33350-33367 | This study |
| 983 | pCT008 (F) | CATTGTATCTATCTTGTGGG | pCT pCT008-pCT009 region | 428 | 3665-3684 | This study |
| 984 | pCT009 (R) | GCATTCCAGAAGATGACGTT | pCT pCT008-pCT009 region | 428 | 4074-4093 | This study |

Primer ISEcp1B can be paired with primer ISEcp1A (527bp) or with primer traK (1037bp). F, forward primer. R, reverse primer.

Table 2.7. Primers used in construction of mutant plasmids and conformation of recombination

| pCT gene | Description | Lab number | Primer sequence | Amplicon size (WT/inactivation) |
|-------------------------------|---|------------|---|---------------------------------|
| <i>bla_{CTX-M-14}</i> | Confirmation forward | 819 | ATGGTGACAAAGAGAGTGCAAC | 876/1764/2361* |
| | Confirmation reverse | 820 | TTACAGCCCTTCGGCGATG | 876/1764/2361* |
| | Recombineering 1 (<i>aph</i>) | 823 | TTTATGCGCAGACGAGTGCGGTGCAGCAAAAGCTGGCGGCGTGAGGCTGGAGCTGCTTC | 1574 |
| | Recombineering 2 (<i>aph</i>) | 824 | CGGCCAGATCACCGCAATATCATTTGGTGGTGCCGTAGTCGGGGAATTAGCCATGGTCCAT | 1574 |
| | Recombineering 1 (<i>aph-gfpmut2</i>) | 1038 | TTTATGCGCAGACGAGTGCGGTGCAGCAAAAGCTGGCGGCCAGGAGTCCAAGCGAGCTCT | 2433 |
| | Recombineering 2 (<i>aph-gfpmut2</i>) | 1039 | CGGCCAGATCACCGCAATATCATTTGGTGGTGCCGTAGTCGAGGAGAGCGTTACCCGACAA | 2433 |
| Sigma factor | Confirmation forward | 868 | ACAGCGTCTTCTCGTATCCA | 1289/1675 |
| | Confirmation reverse | 869 | GTTCTTCCAGCTGACGTAAC | 1289/1675 |
| | Recombineering 1 (<i>aph</i>) | 870 | GGAGGGCGTCTCGCTAAAAAACTTACTCAAACACATCAAGTGAGGCTGGAGCTGCTTC | 1574 |
| | Recombineering 2 (<i>aph</i>) | 871 | GCATTACTTTTATTCTCGTGAGACTCAAGGTCATTTCGGTGGGAATTAGCCATGGTCCAT | 1574 |
| <i>rci</i> | Confirmation forward | 875 | AAGGTCATCTGCAGGAGT | 945/1867 |
| | Confirmation reverse | 876 | GTGTCGCAGCAACAATA | 945/1867 |
| | Recombineering 1 (<i>aph</i>) | 877 | GGGGGACATGCCGTATGAATCCTGTTGAACTGGTCCGAAAGTGAGGCTGGAGCTGCTTC | 1574 |
| | Recombineering 2 (<i>aph</i>) | 878 | GCAGTGTCACGACAAACAGCCCGTTTCTGCACCCGACAGTGGAATTAGCCATGGTCCAT | 1574 |
| <i>pilS</i> | Confirmation forward | 1042 | GCGGAAGGAAGTGAGCATAA | 722/2053 |
| | Confirmation reverse | 1043 | CAGTGACATGCTGAAGCAGT | 722/2053 |
| | Recombineering 1 (<i>aph</i>) | 811 | TGGTGACCAGATCAATACAGTTTTTCTTCGGCACATTGCTGTGTAGGCTGGAGCTGCTTC | 1574 |
| | Recombineering 2 (<i>aph</i>) | 812 | AACCTGCAGACAATCGCCACCAAAATGAAAGCCCAGAAAGGAATTAGCCATGGTCCAT | 1574 |
| ISEcp1 | Confirmation forward | 837 | GCAGGTCTTTTCTGCTCC | 1197/1677 |
| | Confirmation reverse | 872 | CCCTTGACGGATAGTTTTTC | 1197/1677 |
| | Recombineering 1 (<i>aph</i>) | 873 | CAAATAAAATCAAGATGAATCATATAAAGACCATGCTCTGGTGAGGCTGGAGCTGCTTC | 1574 |
| | Recombineering 2 (<i>aph</i>) | 874 | TTCCGACAACCTCATGATTACATATCTTGCGGTTTTGATTGGGAATTAGCCATGGTCCAT | 1574 |
| <i>traXY</i> | Confirmation forward | 1113 | GGAGAGTCCGGTCTGTATGA | 2423/2138 |
| | Confirmation reverse | 1114 | TGCAACCAAGTGTGGTACAG | 2423/2138 |
| | Recombineering 1 (<i>aph</i>) | 1115 | GTATCCTGGTCTGCCTGTTACTGATGAGTACCATTGCAGCGTGAGGCTGGAGCTGCTTC | 1574 |
| | Recombineering 2 (<i>aph</i>) | 1116 | CGGCACAAAACAGCAAAAACAGCAGGAAGTAGAGTGGTGGGGGAATTAGCCATGGTCCAT | 1574 |
| <i>pndACB</i> | Confirmation forward | 1351 | AAGGATTGTGGCGGACAGGA | 486/1288/1751 |
| | Confirmation reverse | 1352 | TGATGACGCACAGGACGGAA | 486/1288/1751 |
| | Recombineering 1 (<i>aph</i> or <i>cat</i>) | 1353 | CCCAGGCGATTTTTTATCAATCAACCCAGGGCCCACTGTGTGTAGGCTGGAGCTGCTTC | 1574/1111φ |
| | Recombineering 2 (<i>aph</i> or <i>cat</i>) | 1354 | ATTGAGGTCAGCCTTCGCAACAATCCGGCGGCAGATGTCCGGGAATTAGCCATGGTCCAT | 1574/1111φ |

Amplicon size, * wild-type pCT/ pCT::*aph*/ pCT::*aph-gfpmut2*; φ *pndACB*::*aph* / *pndACB*::*cat*.

2.5.3 Sequencing of amplimers

PCR amplimers to be sequenced were purified using the QIAquick® PCR purification Kit (QIAGEN, UK, Cat. no. 28104) following the manufacturer's instructions. Pure DNA (2 µl) was added to 4 µl of SDW and 4 µl of 0.8 pm/µl of primer (either forward or reverse). Sequences were determined at the functional genomics department at Birmingham University using a BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems Ltd., USA). The resulting DNA sequences were analysed using homology searches (using the BLAST algorithm) and aligned to known or predicted sequence data using bioinformatics software MEGA 4 (Centre for Evolutionary Functional Genomics, USA).

2.5.4 PCR assays to identify pCT-like plasmids

Using the pCT sequence, primer pairs were designed to amplify novel specific regions of pCT to allow rapid identification of potential pCT-like plasmids in CTX-M-14 producing bacteria (Table 3). PCR was first used to confirm the presence of a *bla*_{CTX-M} group 9 gene. A multiplexed PCR assay was then used to amplify DNA encoding the pCT putative sigma factor, *pilN* gene and shufflon recombinase. Additional primer pairs were designed to a unique region of pCT when compared with other known sequences for amplification across CDSs pCT008-pCT009 and to pCT relaxase gene *nikB* for further discrimination of pCT-like plasmids. Template DNA from CTX-M-14 producing *E. coli* veterinary and clinical isolates (Table 2.3) was prepared by boiling a 100 µl bacterial suspension for 5 minutes to create cell lysates, 1 µl of which was added to PCR ReddyMix Master mixture (Abgene, Epsom UK) as in Section 2.5.1. PCR conditions were 30 cycles of 95 for 30°C seconds, 53°C for 30 seconds and 72°C for 40 seconds using primers in Table 2.6. To determine whether

the pCT *bla*_{CTX-M-14} shares a common insertion site with *bla*_{CTX-M-14} on other plasmids, PCR assays were designed to amplify sequence from *bla*_{CTX-M-14} into both the pCT flanking genes (a pseudogene and *traK* and into upstream and downstream insertion elements *ISEcp1* and *IS903* (Figure 2.1; Table 2.6).

2.5.5 Relaxase (*nikB*) typing and phylogenetic analysis

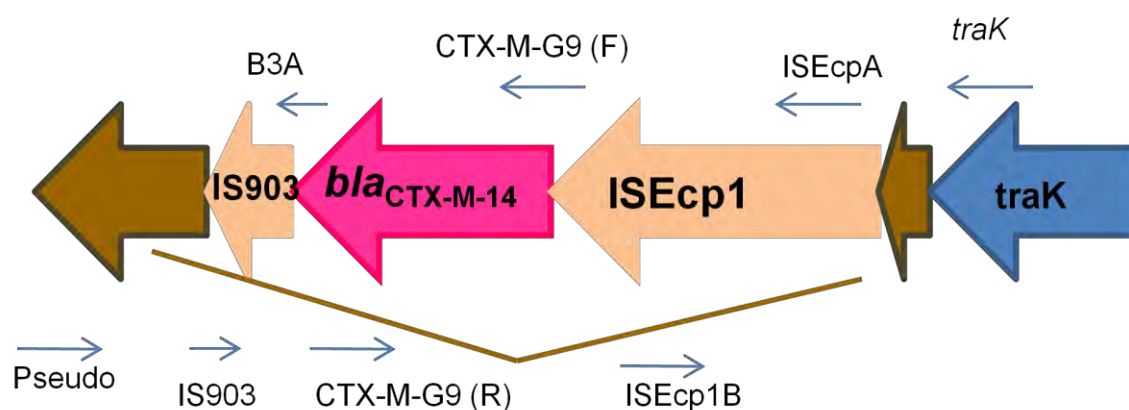
The categorisation and phylogenetic analysis of plasmids by the amplification and sequencing of plasmid encoded relaxase genes has been previously reported for IncI plasmids (Garcillan-Barcia *et al.*, 2009; Valverde *et al.*, 2009). A modified primer pair was designed to this region (*nikB*) using sequence data from pCT and other related sequenced plasmids (Table 2.6, primers 979/980) and used within the pCT detection assay as described in section 2.5.4. Resultant amplicons (indicating the presence of an IncI plasmid) were sequenced using BigDye® Terminator v3.1 cycle sequencing (Applied Biosystems Ltd., USA) at the functional genomics laboratory of the University of Birmingham. Sequences were aligned to the pCT *nikB* sequence using MEGA 4.0 (Tamura *et al.*, 2007). For phylogenetic analysis, a neighbour-joining tree was constructed using the maximum composite likelihood method (Saitou and Nei, 1987). The phylogenetic tree was linearised assuming equal evolutionary rates in all lineages.

2.6 Transfer of pCT into new host strains

2.6.1 Transformation of pCT into *E. coli* DH5α

Pure pCT DNA was transformed into electro-competent *E. coli* DH5α cells (Invitrogen, UK, Cat. no. 18290-015) for analysis and manipulation as this strain

Figure 2.1. Primers used to determine the site of *bla*_{CTX-M-14} insertion on pCT-like plasmids



| Primer 1 | Primer 2 | Expected product size (bp) |
|------------|-------------|----------------------------|
| Pseudo | CTX-M-G9 F | 1636 |
| IS903 | B3A | 887 |
| IS903 | CTX-M-G9 F | 1235 |
| CTX-M-G9 R | CTX-M-G9 F | 876 |
| CTX-M-G9 R | ISEcp1A | 2343 |
| ISEcp1B | ISEcp1A | 527 |
| ISEcp1B | <i>traK</i> | 1037 |

The dark pink arrow represents the *bla*_{CTX-M-14} coding region flanked on pCT by two insertional elements (shown as light pink arrows). External to this region is a pCT specific pseudogene shown as two brown arrows. Primer binding sites for each primer designed to this pCT genomic region are represented by small blue arrows labelled with the name of each primer.

provided a known background with which to analyse the plasmid further. pCT DNA (typically 2 µl) was added to 48 µl of *E. coli* DH5α cells and left on ice for 10 minutes; 2 µl of plasmid pUC19 was also incubated with 48 µl of electro-competent cells to act as a positive control. The cell/plasmid mixtures were transferred to a chilled 2 mm electroporation cuvette (Gene flow, Staffordshire, Cat. no. E6-0060) and electroporated at 1.25 kV (25 µF, 200Ω, 5nm) (EasyjectT electroporator). A negative control containing cells only, without plasmid was also treated in this manner. After transformation the cells were recovered in 1 ml of room temperature LB broth and incubated for 2 hours at 37°C. The recovery mixture was cultured on LB agar plates containing 8 µg/ml of cefotaxime to select for pCT transformants (or 50 µg/ml of ampicillin to select for pUC19 transformants) and plates were incubated aerobically for 24 hours at 37°C and inspected for colonies. Up to ten candidate transformants were sub-cultured onto individual LB agar plates supplemented with 8 µg/ml of cefotaxime and incubated overnight at 37°C. A single colony from each plate was Gram stained and another colony inoculated into 10 ml of LB broth supplemented with 8 µg/ml cefotaxime and incubated overnight at 37°C with shaking. Plasmid DNA was harvested from these overnight cultures using QIAgen® Miniprep kit (QIAgen®, UK, Cat. no. 27106) and visualised using gel electrophoresis. PCR was then used to confirm the presence of a Group 9 *bla*_{CTX-M} gene (Section 2.5.1) in these candidate transformants.

2.6.2 Selection of rifampicin resistant mutants

Rifampicin resistant *E. coli* DH5α and *S. Typhimurium* SL1344 mutants were selected by growing strains on LB agar overnight at 37°C then inoculating 10 ml of LB broth with a single colony of each strain, incubating broth cultures overnight at

37°C with shaking at 225 rpm. Three broth cultures for each strain were then centrifuged at 10,000 x g for 15 minutes to harvest cells. One pellet was re-suspended in 1 ml of LB broth to allow an approximate concentration of 10⁸ cells/ml. The two remaining pellets were re-suspended in 100 µl of LB broth allowing an approximate concentration of 10⁹ cells/ml. Serial dilution 1:10 of the remaining overnight broth culture (10⁷ cells/ml) in LB broth created dilutions containing 10⁶, 10⁵, 10⁴, 10³, 10², and 10¹ cells/ml. From each dilution, 200 µl was cultured in duplicate on LB agar plates supplemented with 100 µg/ml of rifampicin and a LB agar plate with no added antibiotic, and incubated overnight at 37°C. The following day colonies on LB agar plates were counted to determine the viable count (colony forming units/ml) and compared with the number of colonies recorded on rifampicin (100 µg/ml) selection plates. The frequency of resistance was calculated by dividing the number of colonies on each selective plate by the viable count (adjusting for dilutions). Candidate resistant mutants were sub-cultured onto LB agar plates supplemented with 100 µg/ml rifampicin and resistance confirmed by determining the MIC of rifampicin to these strains.

2.6.3 Conjugation of pCT into *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R

pCT was transferred via conjugation from pCT donor *E. coli* DH5α pCT to bacterial hosts *E. coli* J53-2 (I847, rifampicin resistant), *E. coli* 3950 (I823, tetracycline resistant) and *S. Typhimurium* SL1344 rif^R (L1078, rifampicin resistant) for further analysis. Donor and recipient strains were cultured overnight in LB broth at 37°C and shaking and cells from 1 ml of each overnight culture were harvested by centrifugation for 10 minutes at 13,000 x g. *E. coli* DH5α pCT cells were resuspended

in 100 µl LB broth and 50 µl of this suspension was added to recipient strains cells resuspended in 50 µl of LB broth. The donor/recipient mixture was pipetted onto a 0.45 µm/ 25 mm nylon membrane filter (Millipore, UK, Cat. no. HNWP02500) placed on a LB agar plate, and incubated for 12 hours at 37°C. After this time the filter was removed and aseptically placed into a universal tube containing 1 ml of fresh LB broth. Cells were liberated from the filter by vortexing, then diluted appropriately in phosphate buffered saline (PBS) (Sigma, UK, Cat. no. D8537) to isolate single colonies. The mixture was then spread onto LB agar containing 8 µg/ml cefotaxime to select for pCT, and either 100 µg/ml of rifampicin or 50 µg/ml of tetracycline to select for the recipient strain, and incubated for 24 hours at 37°C. As a control 100 µl of each donor and recipient overnight culture were spread on antibiotic containing agar to quantify the frequency of spontaneous mutation. Candidate colonies were also sub-cultured onto XLD agar to allow the identification of any randomly selected rifampicin resistant donor mutants. PCR was used to confirm the presence of a Group 9 *bla*_{CTX-M} gene in these putative transconjugants as in Section 2.5.1.

2.7 Further characterisation of pCT-like plasmids

Isolates identified as carrying a pCT-like plasmid using the PCR assay described in 2.5.4 were grown overnight in LB broth and transformed into *E. coli* DH5α electro-competent cells (Invitrogen, UK, Cat. no. 18290-015) as described in section 2.6.1 for further analysis of these plasmids (Appendix 2). The MIC of a range of antibiotics (Table 2.5) required against these pCT-like plasmid transformants were determined as in Section 2.2 and the ability of these plasmids to conjugate was examined as in Section 2.6.3 from donor *E. coli* DH5α to *E. coli* recipient strain DH5α rif^R.

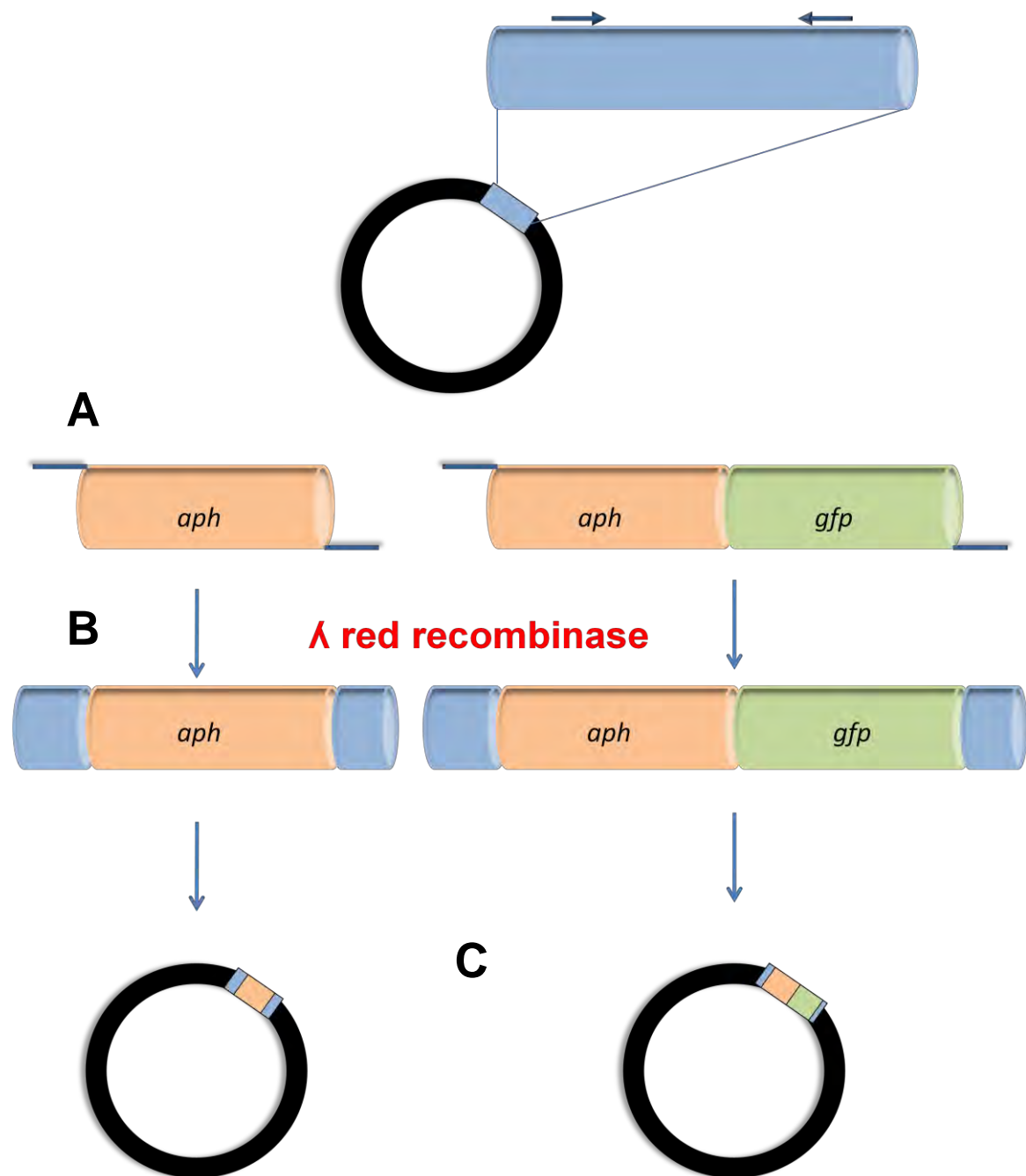
2.8 Inactivation of genes on pCT

A method was developed from the protocol used by Sharan and colleagues (2009) to allow specific inactivation of genes on pCT (Figure 2.2). This method was used to selectively inactivate genes on plasmid pCT by transforming pCT into an intermediate *E. coli* SW102 host strain which encodes a chromosomal λ -red recombinase (Table 2.2) creating strain SW102 pCT (I879).

2.8.1 Design and generation of the DNA integrative cassette with the antibiotic resistance selective marker

For each pCT gene region to be inactivated a primer pair was designed using the PRIMER v.2.00 software package (Scientific and Education Software, UK) with 40 bp of identity to the outermost regions of the pCT target gene and 20 bp homologous to the antibiotic resistance determinant to be amplified (either *aph* or *cat*) (Table 2.7). Kanamycin resistance gene *aph* was amplified from a lysate of *S. Typhimurium* SL1344 *tolC::aph* (L109) and the chloramphenicol resistance gene *cat* was amplified from a lysates of L828 *tolC::cat* (L829) by adding 2 μ l of DNA suspension to a 50 μ l reaction consisting of 45 μ l of Reddymix (ThermoScientific, Waltham, USA, Cat. no. Ab-0575); 1 μ l of forward primer; 1 μ l of reverse primer and 1 μ l of nuclease free water. PCR was as follows, an initial denaturing step at 95°C for 5 minutes followed by 35 cycles of 95°C of denaturation for 30 seconds; annealing at 50°C for 30 seconds and elongation at 72°C for 120 seconds. This was followed by a final extension step at 72°C for 10 minutes. The PCR amplimers were purified using the QIAgen® PCR purification Kit (Qiagen, UK, Cat. no. 27106) and eluted in 30 μ l of nuclease free water. The size of the purified PCR amplimer was determined by electrophoresis of 5 μ l of the sample on a 1% agarose gel.

Figure 2.2. Inactivation of selected pCT genes by homologous recombination with a PCR amplified DNA product.



The inactivation of a selected plasmid gene. A) Primers with 5' 40bp homology to the plasmid gene of interest and 20bp designed to amplify either *aph* or *aph-gfpmut2* were designed. B) PCR amplimers were transformed into strain SW102 already containing plasmid pCT. C) Activation of the λ -red recombinase facilitated the recombination of PCR amplimers with the plasmid gene. This resulted in the removal of genetic material from the plasmid gene and replacement with either *aph* or *aph-gfpmut2*.

2.8.2 Design and generation of the DNA integrative cassette encoding *gfp*

For amplification of the *aph-gfpmut2* construct on plasmid pUA66*pacpP*, plasmid DNA was extracted from strain *S. Typhimurium* L1019 using the QIAgen® Miniprep kit (QIAgen, UK, Cat. no. 27104). Plasmid DNA was then incubated with restriction enzymes *SpeI* (Promega, UK, Cat. no. R6591) and *PstI* (Fermentas, UK, Cat. no. ER1871) in Fermentas Buffer Tango (Fermentas, UK, Cat. no. ER1871) at 37°C for 4½ hours and 65°C for 20 minutes. Primers designed had 20 bp homology to the template pUA66*pacpP* and 40 bp sequence homology to the outermost regions of the target gene *bla*_{CTX-M-14} at the primer 5'-end (Figure 2.2 A, Table 2.7). PCR was carried out as in section 2.8.1 using the digested plasmid as the DNA template. Resulting amplicons consisting of both *aph* and *gfp* were purified using the QIAgen® PCR purification Kit (Qiagen, UK, Cat. no. 27106) and visualised using electrophoresis on a 1% agarose gel.

2.8.3 Recombineering and selection of mutant plasmids

A colony of SW102 pCT (Table 2.2) was placed in 10 ml of LB broth and incubated with shaking overnight at 32°C. From this overnight culture 500 µl was used to inoculate two 35 ml LB broths and shaken at 200 rpm at 32°C until the optical density of the culture reached mid-logarithmic phase ($OD_{600} = 0.5-0.6$). One culture was placed in a shaking water-bath (200 rpm) at 42°C for 15 minutes in order to activate the λ -red recombinase. The other culture remained at 32°C for the same period to act as a control. Both flasks were then immediately placed in an ice bath and repeatedly mixed by hand for a further 10 minutes to cease production of the enzyme. In order to transform these cells with the PCR amplicon for recombination, the bacteria were

made competent using 15% glycerol (150 ml of glycerol in 850 ml H₂O). Cells were harvested by 4°C centrifugation at 2250 x *g* for 15 minutes and resuspended in 30 ml of ice cold 15% glycerol to wash. This process was repeated twice and the resulting pellet of bacterial cells resuspended in 1ml of 15% glycerol. Centrifugation was then repeated at 13,000 x *g* in a micro-centrifuge at 4°C. The harvested cell pellet was washed twice in 15% ice cold glycerol before re-suspending in a volume of 400 µl of glycerol. For electroporation of the PCR amplicon into the newly competent cells (both control and λ -red recombinase activated), DNA was added to cell suspension to make up a volume of 50 µl and left on ice for five minutes. 1 µl of plasmid pUC19 was also added to 49 µl of competent cells (both control and activated) to assess the competence of the cells. The cell/plasmid mixtures were then transferred to a chilled 2 mm electroporation cuvette (Gene flow, Staffordshire, Cat. no. E6-0060) and electroporation was carried out at 1.25 kV (25 µF, 200Ω) (EasyjectT electroporator). A negative control containing bacterial cell suspension only, without plasmid or PCR amplicon was also treated in this manner. After electroporation the cells were suspended in 1 ml of pre-warmed LB broth and incubated for three hours at 32°C. 300 µl of recovery mixture was transferred onto LB agar plates containing 50 µg/ml of kanamycin for inactivation of *bla*_{CTX-M-14}; 50 µg/ml of kanamycin and 8 µg/ml of cefotaxime for inactivation of the other genes with the *aph* construct, and 50 µg/ml of chloramphenicol if inactivating with *cat*. Kanamycin (or chloramphenicol) resistance was used to select for mutant plasmids where recombination had taken place. Ampicillin (50 µg/ml) was also used to select for pUC19 transformants. All agar plates were incubated aerobically at 32°C for 48 hours and then inspected for bacterial colonies. Colonies were sub-cultured onto fresh LB agar containing the appropriate

antibiotic. Plasmid DNA was then harvested using the QIAgen® Miniprep kit (Qiagen, UK, Cat. no. 27106) and electroporated into electro-competent *E. coli* DH5α cells (Invitrogen, UK, Cat. no. 18290-015) as described in section 2.6.1.

2.8.4 Confirmation of gene inactivation

To differentiate between wild-type pCT and mutant pCT which had undergone homologous recombination primers external to the insertion site (confirmation primers) were used to amplify the region across the deletion/insertion site (Table 2.7), yielding a different sized amplicon when compared to wild-type pCT in each case. PCR was carried out as described in section 2.5.1. and visualised as in 2.5.2. PCR that resulted in an amplicon of the expected size (Table 2.7) were purified using QIAquick® PCR purification Kit (Qiagen, UK, Cat. no. 28104) following the manufacturer's instructions and DNA sequenced and analysed as in Section 2.5.3.

In the case of *bla*_{CTX-M-14}::*aph* (pCT2) both mutant and wild-type plasmid were detected by PCR after electroporation into *E. coli* DH5α. Each day for five days a colony was spread onto a fresh LB agar plate containing 50 µg/ml of kanamycin and incubated at 37°C. Isolated colonies were each day checked by PCR for the presence of wild-type pCT. After five days only the mutant plasmid could be detected using PCR and colonies were unable to grow in the presence of cefotaxime (in broth and on supplemented LB agar).

2.8.4.1 *Detection of β-lactamase production*

Strains DH5α pCT2 and DH5α pCT2b (Table 2.4) were also examined for the production of β-lactamases using a synthetic indicator nitrocefin (Montgomery *et al.*, 1979). Strains to be tested were grown aerobically overnight in 10 ml of LB broth at 37°C with shaking at 225 rpm. Bacterial cells were harvested from an overnight

culture by centrifugation and re-suspended in 200 µl of LB broth. An MSE Soniprep (Sanyo Biomedical, UK) was used to lyse cells within the suspension by sonicating for a total of two minutes (4 x 30 second pulses with 3 x 30 second intermissions with no pulse in between) to prevent overheating which may cause protein denaturation. Sonicated preparations were then added in a volume of 50 µl to wells in a 96 well round bottomed microtitre tray (Fisher Scientific, Loughborough, Cat. no. FB56412). Freshly made nitrocefin (10 µl in a concentration of 500 µg/ml) (Fisher Scientific, UK, Cat. no. SR112C) was added to each sonicate (to a final concentration of 83 µg/ml) and observed. A colour change from yellow to red within five minutes demonstrated a β -lactamase producing strain.

2.8.5 Transfer of pCT mutant plasmids pCT2-6 and pCT8 to bacterial host strains *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R

The pCT mutant plasmids pCT2-6 and pCT8 were transferred to host strains *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R for further study using the method in Section 2.6.3 (Table 2.4). Selection of strains containing *bla*_{CTX-M-14} inactivated plasmids was on LB supplemented with 50 µl/ml kanamycin, selection for transconjugants containing the other mutant plasmids was on LB agar supplemented with 50 µg/ml of kanamycin and 8 µg/ml of cefotaxime with the appropriate antibiotic for the recipient strain. Transconjugants from each pairing were grown on XLD to ensure pCT transfer rather than spontaneous mutation in the donor strain. Colonies were also checked using PCR for the expected insertion within target genes to ensure the right pCT plasmid had been transferred.

2.9 Characterisation of plasmid stability and transfer

2.9.1 Plasmid stability *in vitro*

Plasmid persistence was measured by assessing the percentage of cells which lose the plasmid within an isogenic culture over time. From a 10 ml overnight LB broth culture of each strain, 100 µl was used to inoculate 100 ml of 'pre-warmed' LB broth which was incubated at 37°C with shaking at 180 rpm. Every two hours for 12 hours a 100 µl sample was removed to determine the total viable count. At hours 12, 24, 48 and 72 hours 100 µl of culture was used to seed 100 ml of fresh 'pre-warmed' LB broth. A further viable count was taken at 24, 48, 72 and 96 hours. Those plates containing between 20-200 colonies were replica plated using sterile velveteen squares onto an antibiotic free plate and agar containing either 8 µg/ml cefotaxime for wild-type pCT containing strains, 50 µg/ml of kanamycin for pCT2 and pCT2b (*bla*_{CTX-M-14}::*aph/gfpmut2*) containing strains or 8 µg/ml cefotaxime and 50 µg/ml of kanamycin for strains containing pCT3-9. Colonies growing on the antibiotic free plate but not on the antibiotic selection plate indicated the proportion of bacteria to have lost plasmid.

2.9.2 Plasmid conjugation frequencies

Conjugation frequencies were measured for pCT and pCT mutant plasmids (pCT2-9) from donor strain *E. coli* DH5α (Table 2.1 and 2.4) to recipient strains *E. coli* J53-2 (I847, rifampicin resistant), *Salmonella enterica* serovar Typhimurium SL1344 rif^R (L1078, rifampicin resistant) and *E. coli* 3950 (I823, tetracycline resistant). In addition the pCT conjugation frequency was measured from donor *E. coli* C159/11 (I753) to various recipients and from donor DH5α pCT to recipient *E. coli* DH5α rif^R (I754,

rifampicin resistant). Both donor and recipient strains were grown overnight in 10 ml of LB broth at 37°C with shaking at 225 rpm. Overnight cultures were used to seed 30 ml of fresh LB broth at a 4% inoculum and cultures were grown at 37°C at 225 rpm to an OD₆₀₀ of 0.6 and adjusted if necessary. Viable counts were performed at this stage to establish the ratio of donor to recipient strains.

To measure the conjugation frequency in liquid, 1 ml of adjusted recipient culture and 500 µl of donor culture were added together in a universal tube and incubated at 37°C for 3 hours with shaking at 180 rpm. For solid surface conjugation, cells were harvested by centrifugation from 1 ml of adjusted donor and recipient culture. Donor cells were resuspended in 100 µl of fresh LB broth and recipient cells resuspended in 50 µl of LB broth; 50 µl of each were then mixed and placed on a 0.45 µm/ 25 mm nylon membrane filter (Millipore, UK, Cat. no. HNWP02500) on an LB agar plate and incubated at 37°C. After 3 hours the filter was removed into a universal tube and 1 ml of fresh LB broth added. Cells were liberated from the filter by vortexing. When measuring the conjugation frequency in the presence of sub-inhibitory concentrations of cefotaxime, the antibiotic was either added directly to the LB broth to a final concentration of 0.003 µg/ml or 0.006 µg/ml for liquid conjugation, or for conjugation on a solid surface, the appropriate volume of cefotaxime was added to LB agar on which filters were placed.

Conjugation mixtures from both filter and liquid matings were diluted appropriately to isolate single colonies, and plated onto LB agar containing either 50 µg/ml of tetracycline or 100 µg/ml of rifampicin to select for the recipient strain and 8 µg/ml cefotaxime (for selection of pCT), 50 µg/ml of kanamycin (for selection of pCT2 and pCT2b), both cefotaxime and kanamycin for selection of pCT3-8, and 20 µg/ml of

chloramphenicol for selection pCT9. Conjugation frequencies were measured a minimum of three times and calculated using the formula:

Conjugation frequency =

$$\frac{\text{Median number of transconjugants}}{\text{Median number of recipients X (Donor viable count/Recipient viable count)}}$$

Unpaired Student's t-tests were used to determine any significant changes in conjugation frequency, determined as a value where *P* was less than 0.05.

2.10 Effect of pCT and mutant pCT upon host phenotype

2.10.1 Bacterial host growth rate

The growth kinetics of each bacterial strain were measured by monitoring the optical densities of broth cultures over time. An overnight culture of each strain was adjusted in LB broth to contain approximately 10^5 cells/ml and a 200 µl aliquot of adjusted culture (three biological and four technical repeats) was placed in a 96 well round bottomed microtitre tray (Fisher Scientific, Loughborough, Cat. no. FB56412). Plates were incubated at 37°C with shaking in double orbital mode, 3 mm movement at 200 rpm with additional shaking for 3 seconds before each cycle using a FLUOstar OPTIMA (BMG Labtech, UK) and the optical density measured at 600 nm every 10 minutes. The generation times of each strain were calculated by comparing the increase in optical densities during mid-logarithmic phase using the formula

$$\frac{\text{Time}}{3.3 (\log_{10}[X_t - X_o])}$$

where X_o is an approximation of the number of cells, calculated from the optical density ($OD_{600} 0.1 = 100,000$ cells) at the start of the experiment, and X_t is the

estimated number of cells calculated from the optical density at the end of the time period. The generation times of strains were compared using an unpaired Student's t-test where a P value of <0.05 was considered a significant difference.

2.10.2 Biofilm formation assays

The ability of strains to form a biofilm was evaluated using two methods. Crystal violet staining was used to quantify the amount of biofilm produced and culture on LB agar containing Congo red was used to qualitatively assess the extracellular biofilm matrix produced.

2.10.2.1 *Crystal violet staining*

Overnight LB broth cultures of strains were grown at 37°C with shaking at 225 rpm and diluted in LB broth without NaCl to an optical density of 0.1 (OD₆₀₀ nm). A 200 µl aliquot of adjusted cultures were dispensed into a 96 well microtitre tray (Fisher Scientific, Loughborough, Cat. no. FB56412) and incubated for 48 hours at 30°C with gentle agitation (using a tilting platform shaker). Any biofilms formed were washed in sterile distilled water then stained by the addition of 200 µl of 1% crystal violet to each well for 15 minutes. The crystal violet was then removed from each well and the biofilms washed again using sterile distilled water. The crystal violet retained by any biofilm present was solubilised using 70% ethanol and quantified by measuring the optical density (OD₆₀₀) of the ethanol in each well using the FLUOstar OPTIMA (BMG labtech). Three biological and four technical repeats were examined for each strain. Values were blank corrected to wells containing LB broth only and the mean optical density recorded for each strain was compared using an unpaired Student's t-test. A significant difference in biofilm produced was determined as a P value less than 0.05.

2.10.2.2 *Culture on agar containing Congo red*

Overnight bacterial cultures of each strain grown in LB broth at 37°C with shaking at 225 rpm were diluted 1:10,000 in PBS (Sigma, UK, Cat. no. D8537). A 5 µl aliquot of the diluted culture was dispensed onto LB agar without salt and supplemented with 40 µg/ml of Congo red (Sigma Aldrich, UK, Cat. no. C6277-25G), and left to dry. Plates were then incubated at 30°C for a minimum of 48 hours. Red dry and rough colonies were observed for strains producing cellulose but not curli; smooth and white colonies indicated strains producing no curli or cellulose.

2.10.3 Settle assay to determine the aggregative ability of strains

Bacterial strains +/- pCT and the pCT mutants were grown overnight in LB broth at 37°C with shaking at 225 rpm. Cultures were secured and left stationary on the bench for twelve hours to 'settle'. At the beginning of each experiment and every hour thereafter, a 100 µl aliquot was taken from the very surface of each culture. This was diluted in 900 µl of fresh broth and the optical density at 600 nm was recorded. In each experiment *E. coli* 042 (I845) was used as a positive control. Each optical density (OD₆₀₀) value was shown as a percentage of the initial OD₆₀₀.

2.10.4 Imaging of cells

For imaging of bacterial strains using scanning electron microscopy, cells were prepared in two ways. To visualise cells from a solid surface each strain was cultured overnight on LB agar at 37°C. A colony from each strain was then suspended in 10 µl of PBS (Sigma, UK, Cat. no. D8537) on a glass cover-slip, left to dry and then fixed by submersion of the glass side in 2.5% Glutaraldehyde and 0.1 M Phosphate buffer for 1 hour. Alternatively, overnight LB broth cultures were used to seed 30 ml of fresh

LB broth which were cultured to late-logarithmic phase ($OD_{600} \sim 0.8-0.9$). These cells were used to visualise bacteria grown in liquid as 1 ml of each culture was placed into 3 ml of 2.5% Glutaraldehyde and 0.1 M Phosphate buffer, also for 1 hour. Samples were then kindly prepared using dehydration, and mounted using platinum by Theresa Morris (School of Metallurgy and Materials, University of Birmingham). Imaging of bacterial cells used a FEI/ Philips XL30 FEG ESEM (Philips).

2.10.5 Effect of pCT and the pCT2-9 upon the ability of bacterial host strains to cause infection

The contribution of wild-type pCT and the pCT mutant plasmids pCT2-9 to the bacterial host strain's ability of to cause infection were assessed using two models of infection. The first measured the ability of *S. Typhimurium* SL1344 (+/- rif^R ; +/- plasmids) to adhere to, and invade, human embryonic intestinal cells (INT-407) within a monolayer. The second model measured the rate at which nematode *C. elegans* were killed when fed on cultures of *S. Typhimurium* SL1344 (+/- rif^R) and *E. coli* 3950 containing either no plasmid, wild-type pCT or the pCT mutant plasmids.

2.10.5.1 *Tissue culture model*

A Human embryonic intestine cell line INT-407 was kindly supplied by R. La Ragione at the Animal Health and Veterinary Laboratories Agency (AHVLA), Addlestone, Surrey for investigation of *S. Typhimurium* pathogenicity by previous members of the ARG research team (Buckley *et al.*, 2006; Blair *et al.*, 2009). The methods were based upon those described by Dibb-Fuller and colleagues (Dibb-Fuller *et al.*, 1999). Each assay was repeated a minimum of three times and each repeat contained four technical repeats.

2.10.5.1.1 Preparation of INT-407 tissue culture cells

INT-407 monolayers were grown in 250 ml tissue culture flasks (BD Falcon™, US, Cat. no. 353136) in complete tissue culture medium containing Minimum Essential Medium (MEM) (Sigma-Aldrich, UK, Cat. no. M2279) and supplemented with 10% heat inactivated foetal calf serum (Invitrogen, UK, Cat. no. 10108-165), 1% non-essential amino acids (NEAA) (Sigma, UK, Cat. no. M7145), 1% L-glutamine (Sigma, UK, G7513) and 50 µg/ml gentamicin (Sigma, UK, Cat. no. G1397). For use in the adhesion and invasion assays monolayers were prepared in 24 well flat bottomed tissue culture plates (BD Falcon™, US, Cat. no. 353047). Each well was seeded with approximately 10^5 cells and incubated for 48 hours at 37°C and 5% CO₂. Before the infection assays the prepared monolayers were washed with 1 ml of pre-warmed Hanks Balanced Salt Solution (HBSS) (Sigma, UK, Cat. no. H9269) by pipetting 1 ml into each well and then removing and discarding the media, three times for each well to remove residual medium and antibiotic.

2.10.5.1.2 Preparation of bacterial strains for the tissue culture assays

Bacterial strains were grown overnight in 10 ml of LB broth containing appropriate antibiotics to ensure maintenance of the plasmid at 37°C and shaking at 225rpm. Cells were harvested from each culture by centrifugation at 2250 x *g* for 10 minutes at room temperature and the supernatant discarded. Bacterial cells were washed by re-suspending the pellet in 10 ml of sterile PBS (Sigma, UK, Cat. no. D8537) and centrifugation at 2250 x *g* for a further 10 minutes at room temperature. Harvested cells were then re-suspended in approximately 10 ml of sterile PBS and adjusted to an optical density of 1.23 at 540 nm with PBS (approximately 1×10^7 CFU/ml). The bacterial cultures were then diluted 1:20 in inoculation medium and viable counts

were performed on this diluted bacterial culture. Inoculation media consisted of MEM medium supplemented with 1% L-glutamine (Sigma, UK, Cat. no. G7513) and 1% NEAA (Sigma, UK, Cat. no. M7145).

2.10.5.1.3 Association assays

To quantify the number of bacteria of each strain which associated with the INT-407 cells within the monolayer, 1 ml of the prepared bacterial suspension was inoculated into each well and incubated for two hours at 37°C and 5% CO₂. To remove all non-adherent bacteria, monolayers were washed four times with 1 ml of pre-warmed HBSS. Monolayers were disrupted by adding 1 ml of sterile 1% Triton-X 100 (Sigma, UK, Cat. no. X100) and a magnetic flea to each well and placed on a magnetic stirrer for 10 minutes. Serial 1:10 dilutions were made from each tissue culture well and three 20 µl drops of each dilution were plated onto LB agar as described by Miles *et al.*, (1938). Agar plates were incubated overnight at 37°C and counted to determine the number of colony forming units (CFU) per millilitre.

2.10.5.1.4 Invasion assays

To quantify the number of bacteria of each strain which invaded the INT-407 cell, invasion assays were carried out in parallel with the association assays using the same prepared bacterial suspension to inoculated each well. Monolayers were incubated for two hours at 37°C and 5% CO₂ and washed four times with 1 ml of pre-warmed HBSS as previously described. Two millilitres of complete tissue culture medium containing 100 µg/ml of gentamicin (Sigma, UK, Cat. no. G1397) was then added to each well to kill bacteria which had not invaded without damaging or penetrating the eukaryotic cells, thus leaving the internal bacteria unharmed. The plate was incubated for a further two hours at 37°C and 5% CO₂. Monolayers were

washed four times with 1 ml of pre-warmed HBSS and then disrupted by adding 1 ml of sterile 1% Triton-X 100 (Sigma, UK, Cat. no. X100), a magnetic flea to each well and placing on a magnetic stirrer for 10 minutes. Serial 1:10 dilutions were made from each tissue culture well and three 20 µl drops of each dilution were plated onto LB agar as described by Miles *et al.*, (1938). Agar plates were incubated overnight at 37°C and colonies counted to determine the number of colony forming units (CFU) per millilitre.

2.10.5.1.5 Statistical analysis

The number of bacteria able to associate and invade tissue culture cells was calculated for each strain by finding the mean CFU/ml (and standard deviation) for each biological replicate. The mean CFU/ml of the three biological repeats was then calculated to give the overall CFU/ml. The level of adhesion was determined by subtracting the mean CFU/ml for invasion from the mean CFU/ml for association. Each value was then expressed as a percentage of that given by the value obtained for the parental strain (either *S. Typhimurium* SL1344 rif^R or *S. Typhimurium* SL1344 rif^R pCT) and a Student's *t*-test was used to determine whether the adhesion and invasion values of each strain were significantly different to that of the parental strains. *P* values of ≤ 0.05 were deemed to be statistically significant. A 95% confidence interval for the mean association of *S. Typhimurium* SL1344 with INT-407 cells was calculated by a previous member of the laboratory. Only data from assays where a value for *S. Typhimurium* SL1344 fell within this range (1.2×10^6 to 1.1×10^7 CFU/ml) were accepted.

2.10.5.2 *C. elegans* model

Bristol N2 *C. elegans* were kindly donated by Dr Robin May at the University of Birmingham for use in this study. Nematodes were routinely cultured on a lawn of *E. coli* OP50 on nematode growth media (NGM) (Appendix 3) and stored at 20°C. Approximately every four days between ten and thirty nematodes were removed and seeded onto a fresh *E. coli* OP50 NGM plate to maintain the culture.

2.10.5.2.1 Preparation of bacterial strains and assay plates

NGM agar was poured into 60 mm Petri dishes (Sterilin, UK, Cat. no. 123) and allowed to dry. Overnight cultures of each bacterial strain grown at 37°C in LB broth were used to seed NGM assay plates by pipetting 20 µl of culture onto the middle of the plate and leaving to dry. Plates were then incubated overnight at 37°C to allow proliferation of the bacterial strains. For each bacterial strain three cultures were used to inoculate twenty plates each to create three biological repeats.

2.10.5.2.2 Preparation of *C. elegans*

A recently starved *E. coli* OP50 NGM plate was washed repeatedly with M9 buffer (Appendix 3) to collect both nematodes and eggs from the plate and liquid made up to 1.5 ml with M9 buffer. Eggs and nematodes were then collected by centrifugation at 3,000 x *g* for 30 seconds and the supernatant was discarded. To kill all nematodes leaving only eggs, 80 µl 5 M sodium hydroxide and 160 µl sodium hypochlorite was added to the pellet and the mixture was shaken vigorously by hand for 2 minutes. M9 was added to make the solution up to 1.5 ml and the mixture was centrifuged at 3,500 rpm for 30 seconds. The pellet was then washed by re-suspending in 1 ml of M9 buffer and centrifuging at 4,500 x *g* five times to remove any trace of sodium hydroxide or sodium hypochlorite. The final pellet was resuspended in 200 µl of M9

buffer and aerated overnight using a Stuart tilting tube roller (Scientific Laboratory Supplies, Nottingham, Cat. no. MIX1980). The next day 200 µl of the mixture was placed around the outside of an *E. coli* OP50 seeded NGM 90 mm agar plate checking for the presence of L1 viable stage nematodes. Plates were incubated at 20°C for 48 hours to allow the nematodes to develop to stage 4 (L4).

2.10.5.2.3 Killing assays

Bacterial killing assays were conducted as previously described by Aballay and colleagues (2001), 60 larval stage 4 (L4) nematodes were used for each biological repeat of the bacterial strains. Twenty nematodes were placed on each assay plate inoculated with the bacterial test strain, incubated at 25°C and scored daily for the number of *C. elegans* which had survived. Each plate was visualised using a Nikon SMZ800 Zoom Stereomicroscope (Nikon Instruments Inc, USA, SMZ-800). Dead nematodes were defined as those which failed to respond to mechanical stimulus, such individuals were removed once identified, and viability counting continued until all the nematodes had died. After each 48 hours of the fertile period, worms were transferred to fresh NGM plates seeded with bacteria from the same overnight culture as in the initial growth using a 'pick' made from 0.25 mm platinum wire (Sigma Aldrich, UK, Cat. no. 349402-250G). A Kaplan Meier estimate was used to determine the probability of *C. elegans* survival over time. Survival curves were then generated by plotting the probability of survival against time. Survival curves were compared using the log rank test and chi-squared analysis to establish whether the difference between two curves was statistically significant (a *P* value less than 0.05 was considered statistically significant).

2.11 Pair-wise competitive growth *in vitro*

Overnight bacterial cultures of the two bacteria strains to be placed in competition were grown at 37°C in LB broth without antibiotic. Both cultures were adjusted using LB broth to an optical density of 1.0 (OD₆₀₀) and 50 µl of each culture added to 100 ml of 'pre-warmed' fresh LB broth for incubation at 37°C shaking at 180 rpm. Competition mixtures were passaged by removing 100 µl of culture at hours 12, 24, 48 and 72 hours to inoculate 100 ml of fresh 'pre-warmed' broth. Viable counts were carried out at the start of the experiment and every two hours for 12 hours, also at hours 24, 48, 72, and in later studies, 96 hours by spreading the competition mixture on LB agar without antibiotic. LB agar plates with 20-200 colonies were replica plated using velveteen squares onto an antibiotic free plate and onto agar supplemented with the appropriate selective antibiotic to distinguish between strains. The number of colonies growing on each type of agar plate were enumerated and revealed the relative proportion of each strain (Figure 2.3). Each competition experiment was carried out on three separate occasions. The number of generations was calculated using the viable count data using the formula

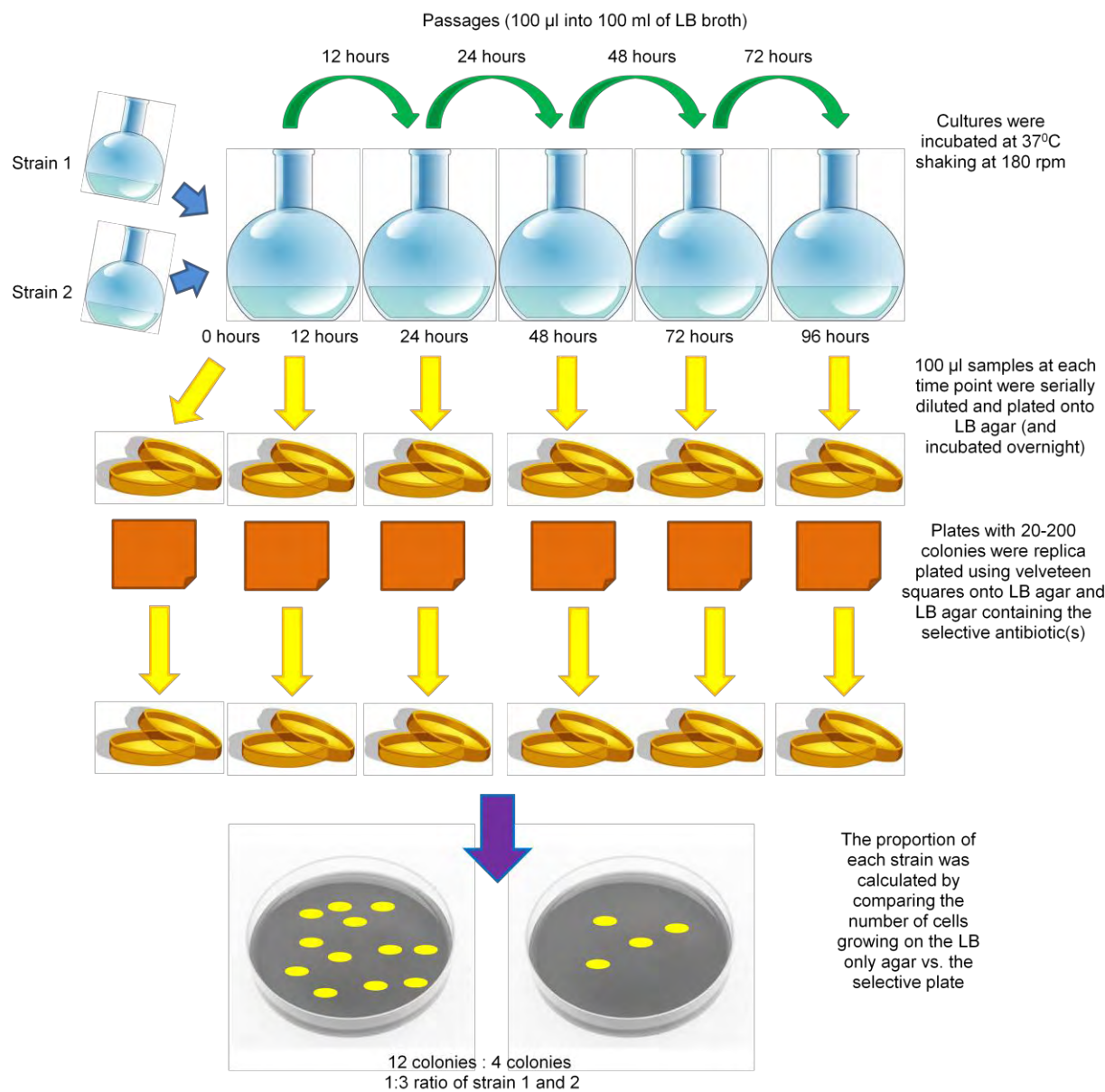
$$\text{Number of generations} = \frac{\log_{10} [X_t] - \log_{10} [X_o]}{0.301}$$

The competition index (CI) was then calculated using the formula,

$$CI = 1 + \frac{\log_{10} [A] - \log_{10} [B]}{\text{number of generations}}$$

Where A is the mean ratio of the strains of the three experiments at the end of the assay, and B is the mean ratio at the beginning of the assay.

Figure 2.3. Typical pair-wise competitive growth method



Chapter 3:

Characterisation and epidemiology of plasmid pCT

3. Characterisation and epidemiology of plasmid pCT

3.1 Background

E. coli isolate C159/11, described by Teale *et al.* (2004) and Liebana *et al.* (2006) was resistant to several different classes of antibiotic. Resistance to third generation cephalosporins was mediated by a *bla*_{CTX-M-14} gene located on an IncK conjugative plasmid (named in the present study, pCT). The Animal Health and Veterinary laboratories Agency (Surrey, UK) showed that an IncK plasmid with an identical restriction fragment length polymorphism (RFLP) profile to pCT had spread throughout the index farm to six different *E. coli* strains, and that pCT could be transferred to *E. coli* and *S. Typhimurium* strains using conjugation (Liebana *et al.*, 2006). In 2008, at the start of the present study, no *bla*_{CTX-M-14} carrying plasmids and only one IncK plasmid had been fully sequenced and annotated. Therefore, little was known about the genetic context of *bla*_{CTX-M-14} outside the proximal insertion elements and even less known about the core genome of IncK plasmids.

3.2 Hypotheses and Aims

Analysis of the complete DNA sequence of pCT would reveal any additional candidate genes, besides *bla*_{CTX-M-14}, which could contribute to the successful spread and persistence of this plasmid. The second hypothesis was that pCT-like plasmids would be found in other geographical locations and other environmental niches.

The aims of this section were to:

- Extract pCT from the natural host strain *E. coli* C159/11, and transfer this plasmid to other bacterial strains for further characterisation and investigation,

- Determine the complete DNA sequence of plasmid pCT and to interrogate the pCT genome by annotation and comparisons to other sequenced plasmids,
- Use this sequencing data to design a molecular diagnostic tool to screen for pCT-like plasmids in CTX-M-14 producing bacteria. By screening isolates collected from different geographical regions the epidemiological distribution of pCT would be explored, thereby establishing its relevance in the dissemination of *bla*_{CTX-M-14}.

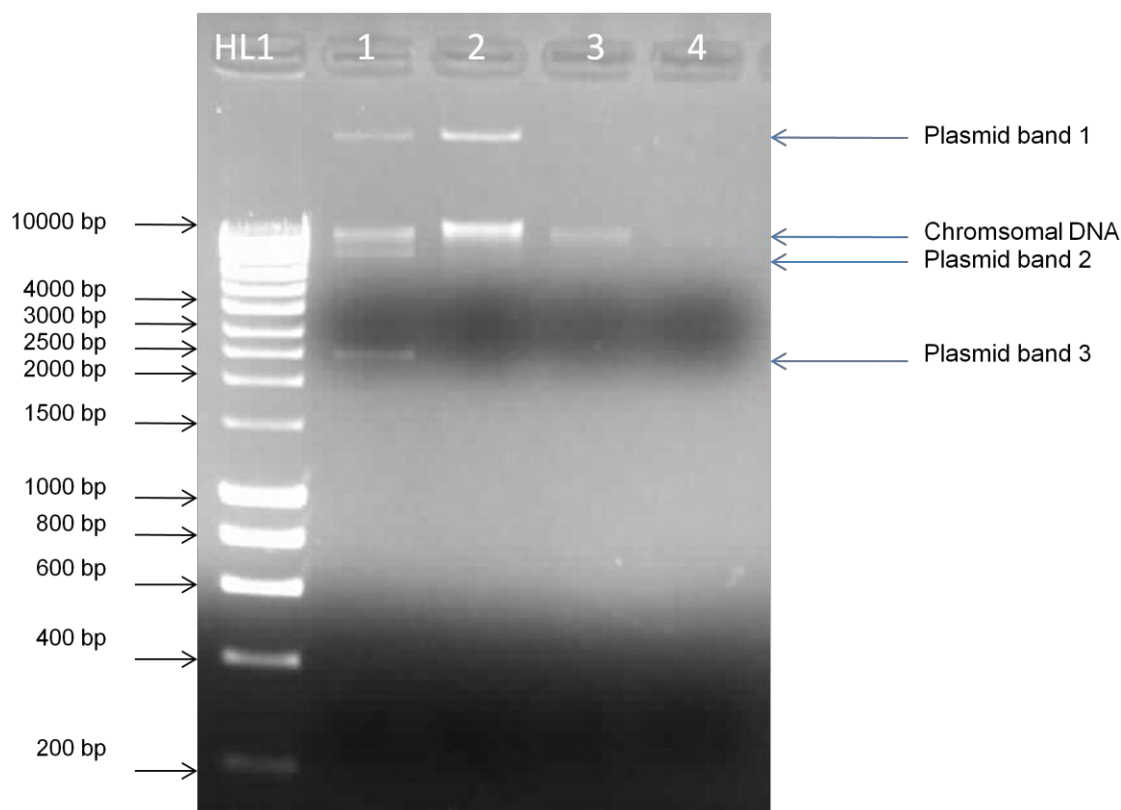
3.3 Preparation of bacterial strains containing pCT

For use in all aspects of this project, plasmid pCT was transferred to three commonly used laboratory host strains *E. coli* DH5 α (l825), *E. coli* J53-2 (l847) and *Salmonella* Typhimurium SL1344 rif^R (L1078) and to the pCT 'cured' natural *E. coli* host strain C159/11 named '3950' (l823).

3.3.1 Transformation of *E. coli* DH5 α with pCT

pCT DNA isolated from wild-type *E. coli* host C159/11 was transformed into *E. coli* DH5 α electro-competent cells. All transformants were confirmed to be *E. coli* using API20E strips and were resistant to cefotaxime. Plasmid DNA was harvested from one putative transformant, and analysed by eletrophoresis on an agarose gel. The extracted plasmid was of a similar size to one of the three plasmids isolated from strain *E. coli* C159/11 (Figure 3.1, plasmid band 1) but absent in the *E. coli* DH5 α parental strain. A specific PCR designed to amplify Group 9 *bla*_{CTX-M} genes and sequencing of the resultant amplicon confirmed the presence of a *bla*_{CTX-M-14} gene in the *E. coli* DH5 α transformant (Figure 3.2; Appendix 1). This strain was named *E. coli* DH5 α pCT and given the laboratory number l755.

Figure 3.1. Plasmid DNA isolated from *E. coli* C159/11 and the transformed *E. coli* DH5α pCT

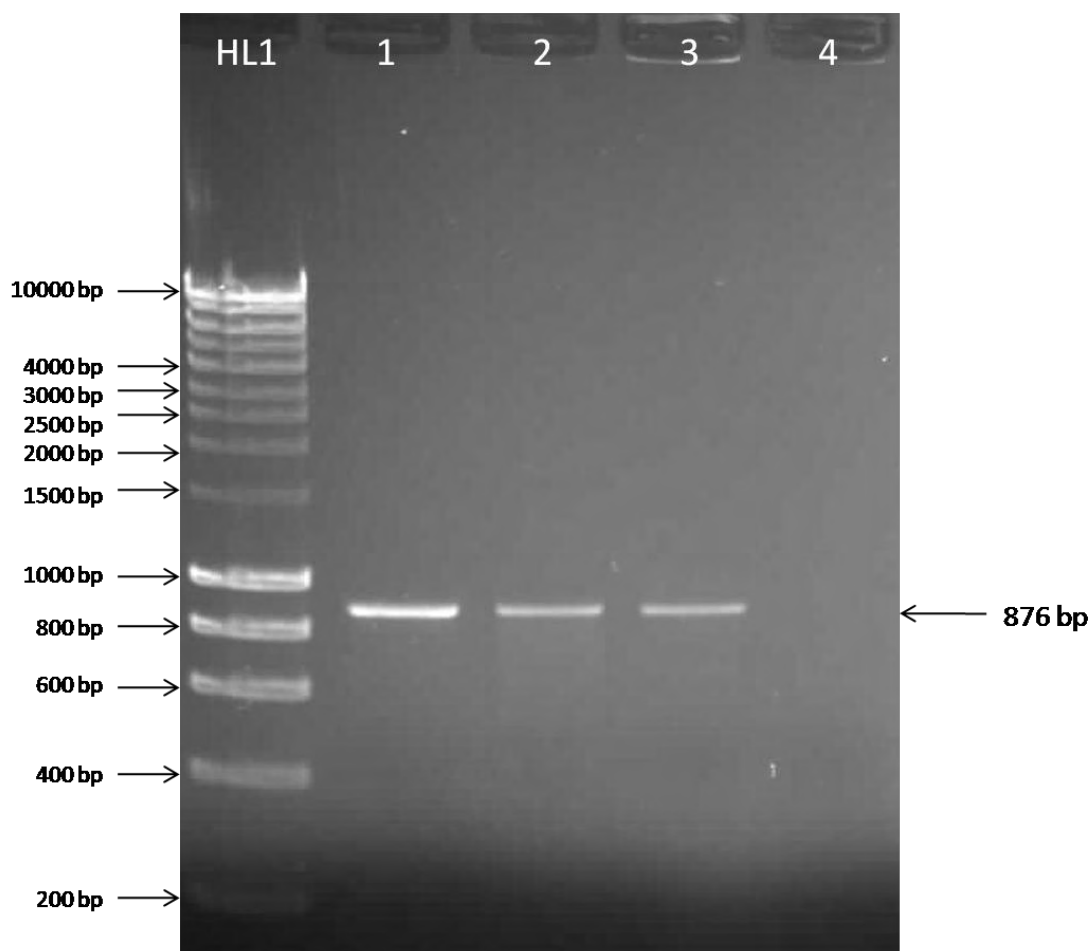


| Lane | Code | Species | Description | Plasmid band present |
|------|------|----------------|-------------------------------------|----------------------|
| HL1 | | | Hyperladder 1 size marker (Bioline) | |
| 1 | I753 | <i>E. coli</i> | C159/11 veterinary isolate (pCT) | 1/2/3 |
| 2 | I755 | <i>E. coli</i> | DH5α transformant (pCT) | 1 |
| 3 | I825 | <i>E. coli</i> | DH5α | |
| 4 | | | Negative control | |

DNA was extracted using the QIAgen® Miniprep kit and analysed by electrophoresis in a 0.7% agarose gel.

Plasmid band 1 shows plasmid pCT DNA, plasmid band 2 and 3 show DNA from two additional plasmids isolated from veterinary *E. coli* isolate C159/11 which were not transformed into *E. coli* DH5α when selecting on agar containing cefotaxime.

Figure 3.2. *bla*_{CTX-M} Group-9 PCR detection in putative pCT containing strains



| Lane | Isolate | Species | Description |
|------|---------|-----------------------|--|
| HL1 | | | Hyperladder 1 size marker (Bioline) |
| 1 | I753 | <i>E. coli</i> | C159/11 Veterinary isolate |
| 2 | I755 | <i>E. coli</i> | DH5α pCT Transformant |
| 3 | L1079 | <i>S. Typhimurium</i> | SL1344 rif ^R pCT Transconjugant |
| 4 | I825 | <i>E. coli</i> | DH5α |

Amplified DNA was analysed by electrophoresis in a 1% agarose gel. The amplification and visualisation of DNA indicated the presence of a *bla*_{CTX-M-9} group gene within the tested strain.

3.3.1.1 Conjugation of pCT into *E. coli* and *S. Typhimurium* strains

A *S. Typhimurium* strain with a chromosomal resistance marker was required to act as a pCT conjugation recipient strain and for further study of pCT in a *S. Typhimurium* bacterial host. Therefore, rifampicin resistant mutants of well characterised laboratory strain *S. Typhimurium* SL1344 were selected using LB agar supplemented with rifampicin (100 µg/ml). The frequency of mutation to a resistant phenotype was calculated as 7.78×10^{-7} per cell (indicative of a single point mutation) and the MIC of rifampicin required against the putative mutants was determined as > 256 µg/ml. A mutant colony was randomly selected and designated *S. Typhimurium* SL1344 rif^R (L1078). Recipient strains *E. coli* J53-2 (I847) and *E. coli* 3950 (*E. coli* C159/11 pCT 'cured'; I823) had chromosomal antibiotic resistance genes conferring resistance to rifampicin and tetracycline respectively.

Conjugation of pCT from donor *E. coli* DH5α pCT (I755) to recipients *S. Typhimurium* SL1344 rif^R, *E. coli* J53-2 and *E. coli* 3950 all produced colonies on selective agar, therefore six putative transconjugant colonies were selected from each experiment for further investigation. Verification of plasmid transfer was carried out by culturing donor, recipients and potential transconjugants on XLD plates. *E. coli* DH5α pCT donor cells grew on XLD as pink colonies (atypical for an *E. coli* K12 strain due to inactivation of *lacZYA*). *Salmonella Typhimurium* SL1344 rif^R was identified as pink colonies with the production of H₂S as a black pigment, and recipient strains *E. coli* 3950 and *E. coli* J53-2 grew as yellow colonies. These differences in phenotype allowed discrimination between transconjugants and spontaneous donor cell mutants. From each conjugation, one randomly selected putative transconjugant was selected and PCR used to confirm the presence of a Group 9 *bla*_{CTX-M} gene

(Figure 3.2). The *S. Typhimurium* SL1344 rif^R pCT transconjugant was designated *S. Typhimurium* SL1344 rif^R pCT (L1079), the *E. coli* J53-2 transconjugant, *E. coli* J53-2 pCT (I855) and the *E. coli* 3950 transconjugant, *E. coli* 3950 pCT (I834).

3.4 Antibiotic susceptibilities of pCT containing host strains

Susceptibility to a range of antibiotics was determined for natural strain C159/11 (pCT) (I753), pCT transformant and pCT transconjugants by measuring the minimum inhibitory concentrations. The range of antibiotics selected were chosen to represent different mechanisms of antibiotic action and to identify the resistance conferred by common plasmid mediated antibiotic resistance genes. According to breakpoints recommended by BSAC guidelines, *E. coli* C159/11 was resistant to the β -lactams ampicillin, cefotaxime and ceftiofur as well as chloramphenicol, tetracycline and ciprofloxacin (Table 3.1). C159/11 (I753) was susceptible to faropenem, gentamicin, erythromycin and rifampicin. The only transferable antibiotic resistance conferred by either the transformation or conjugation of plasmid pCT to the bacterial host strain was to the β -lactams, cefotaxime, ceftiofur and ampicillin (Table 3.1).

3.5 Genomic analysis of plasmid pCT

In order to determine the pCT genome, chain termination DNA sequencing at the Sanger Institute was used to amplify random fragments which, when assembled would reveal the complete plasmid sequence.

3.5.1 Isolation of pCT plasmid DNA for sequencing

High quality and high purity plasmid DNA was required for complete sequencing of pCT. Therefore, pCT was extracted from the *E. coli* DH5 α pCT transformant (I755) using a combination of an alkaline SDS Maxi preparation (Figure 3.3A) followed by

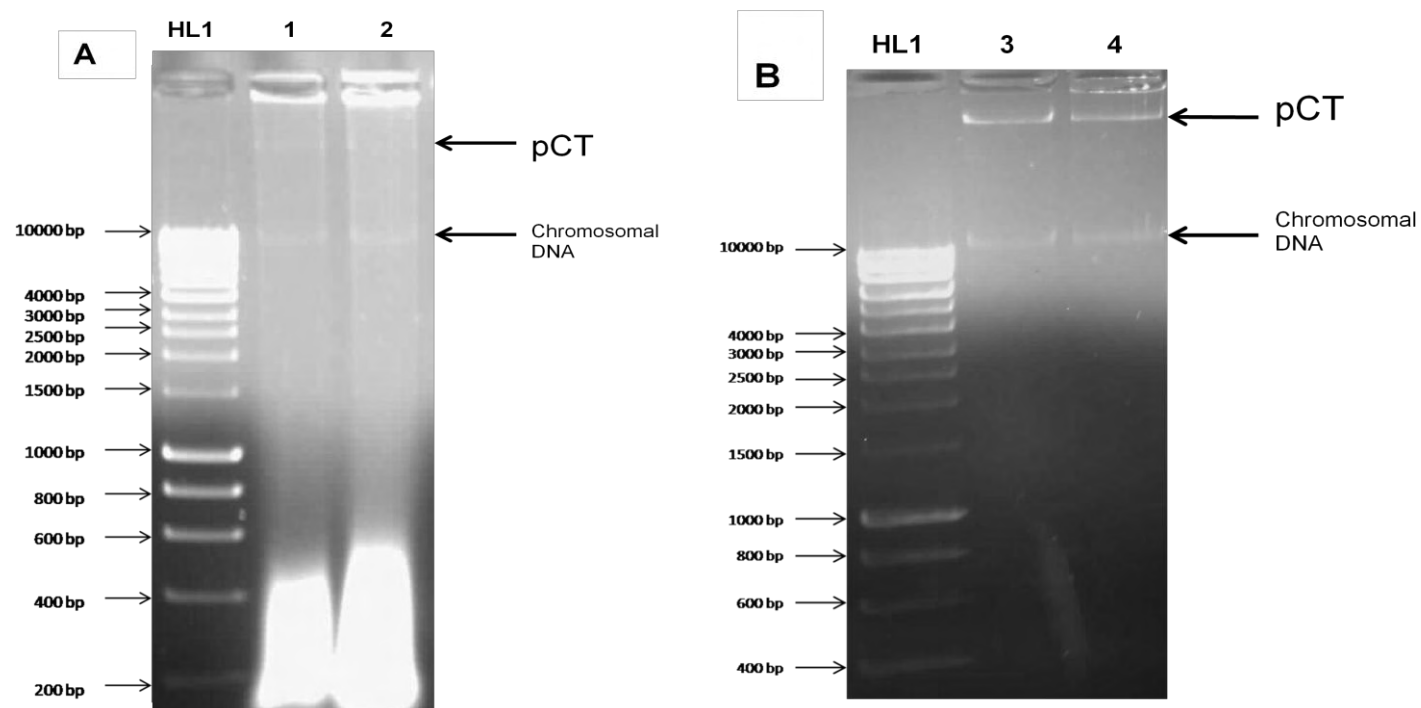
Table 3.1. Minimum inhibitory concentrations of a range of antibiotics to strains with and without plasmid pCT

| Species | Lab code | Strain | Minimum Inhibitory concentration (µg/ml) | | | | | | | | | |
|-----------------------|----------|-----------------------------|--|------------|-----------|------|--------|-----|------|-----|-----|-----|
| | | | CTX | AMP | CFR | FAR | CIP | KAN | GEN | ERY | TET | CHL |
| <i>E. coli</i> | I753 | C159/11 (WT) | 32 | 512 | 64 | 1 | 128 | 2 | 0.5 | 32 | 128 | 32 |
| | I825 | DH5α | 0.03 | 2 | 0.25 | 0.25 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 |
| | I755 | DH5α pCT | 16 | 256 | 32 | 0.5 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 |
| | I847 | J53-2 | 0.06 | 8 | 0.5 | 1 | 0.0015 | 2 | 0.25 | 32 | 2 | 1 |
| | I855 | J53-2 pCT | 16 | 256 | 32 | 1 | 0.0015 | 2 | 0.12 | 32 | 2 | 1 |
| | I823 | 3950 | 0.03 | 8 | 0.5 | 0.5 | 64 | 1 | 0.25 | 32 | 128 | 16 |
| | I834 | 3950 pCT | 16 | 256 | 64 | 0.5 | 64 | 1 | 0.25 | 32 | 128 | 16 |
| <i>S. Typhimurium</i> | L1078 | SL1344 rif ^R | 0.06 | 4 | 1 | 0.5 | 0.03 | 1 | 0.25 | 64 | 2 | 1 |
| | L1079 | SL1344 rif ^R pCT | 64 | 512 | 64 | 0.5 | 0.0015 | 1 | 0.5 | 64 | 2 | 1 |

CTX, cefotaxime; AMP, ampicillin; CFR, ceftiofur; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; ERY, erythromycin, TET, tetracycline, CHL, chloramphenicol; FAR, faropenem.

Bold type MIC values indicate resistance conferred by pCT.

Figure 3.3. Isolation of pCT plasmid DNA



| Lane | Description |
|------|---|
| HL1 | Hyperladder 1 |
| 1 | Plasmid DNA isolated using the Birnboim-Doly Maxi prep; Sample 1 |
| 2 | Plasmid DNA isolated using the Birnboim-Doly Maxi prep; Sample 2 |
| 3 | pCT DNA after alkaline lysis and CsCl gradient centrifugation (Sample 1 and 2 combined) |
| 4 | pCT DNA product after isopropanol and ethanol washes |

Isolated plasmid DNA was analysed using electrophoresis in a 0.7% agarose gel

caesium chloride density gradient centrifugation. The latter procedure was to remove any remaining chromosomal DNA (Figure 3.3B). The DNA isolated after alkaline SDS extraction had extensive chromosomal DNA contamination, as shown at the bottom of the agarose gel as a smear (Figure 3.3A). After caesium chloride density gradient centrifugation (Figure 3.3, Lane 3) the visible chromosomal DNA was removed and a much clearer pCT band >10,000bp was seen. The DNA was cleaned resulting in a more defined plasmid band (Figure 3.3, Lane 4). Spectrophotometric analysis estimated the quantity of DNA used for sequencing in the 46 μ l sample as 40.75 μ g in total (~885 ng/ μ l).

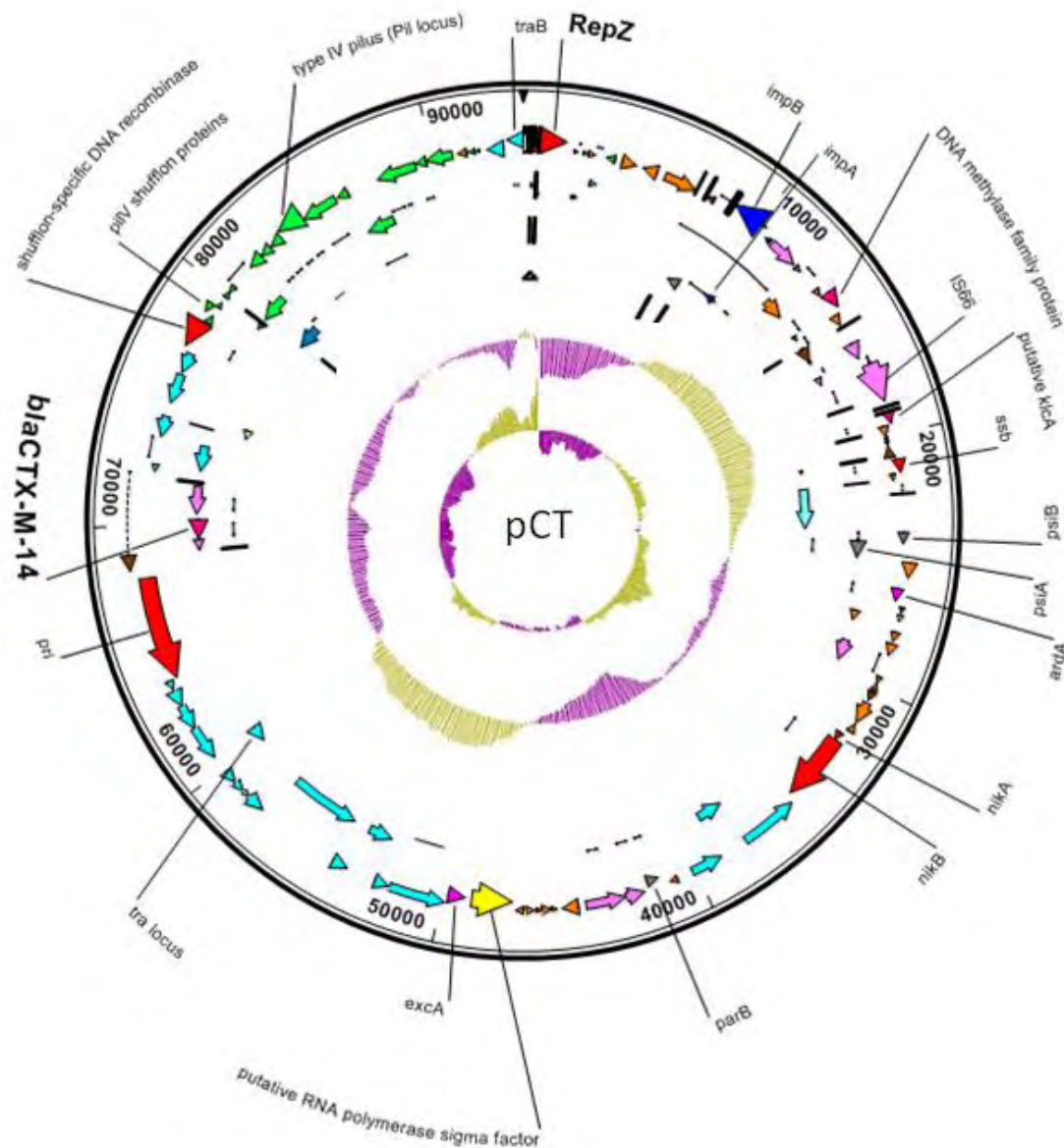
3.5.2 Complete DNA sequencing and annotation

The plasmid DNA sequence was determined at the Sanger Institute using chain termination sequencing to generate 7 contigs. Initial annotation at the Sanger used the 454/Roche Newbler assembly program and Artemis. Further annotation as part of my project was completed using Artemis and BLASTn.

3.5.3 General features of pCT

Plasmid pCT is 93,629b bp in size and has an average G+C content of 52.67%. Within the sequence 115 open reading frames (ORFs) were putatively identified. Of these 115 ORFs, 7% were annotated as pseudogenes due to missing C or N terminals or genomic disruptions; 17% were postulated to encode hypothetical proteins of unknown function; and 76% showed either DNA or protein identity to sequences encoding proteins with known functions. Coding regions included genes for plasmid replication, conjugation and stability along with a number of mobile genetic elements (Figure 3.4 and Table 3.2).

Figure 3.4. Plasmid map of pCT



Circular genomic map of plasmid pCT; coding regions are colour coded as follows, brown, pseudogenes; orange, hypothetic proteins; light pink, insertion sequences; light blue, *tra* locus; green, *pil* locus; dark pink, antibiotic resistance gene; yellow, putative sigma factor; red, replication associated genes. The arrows show the direction of transcription.

The inner circles shows percentage GC (inner) and GC skew (outer).

Table 3.2. Protein coding regions of pCT listed clockwise

| Gene name | ID | Direction of coding | Co-ordinates | No of aa | DNA identity pO26-vir | DNA identity Collb-P9 | Protein Homolog | Function | Notes |
|------------------|---------|---------------------|--------------|----------|-----------------------|-----------------------|-----------------|--|--|
| <i>repY</i> | pCT_001 | → | 610-699 | 29 | 100% | 100% | RepY | Positive regulator for <i>repZ</i> | Leader peptide |
| <i>repZ</i> | pCT_002 | → | 687-1718 | 343 | 100% | 100% | RepZ | Replication initiator protein | |
| | pCT_003 | → | 2050-2199 | 49 | 100% | 95% | | Uncharacterised protein | |
| <i>repA</i> * | pCT_004 | → | 2193-2267 | 128 | 96% | 94% | RepA4 | Replication initiator protein | <i>repA4</i> found in R100 |
| | | | 2267-2440 | | | | | | |
| | pCT_005 | → | 2416-2574 | 51 | 90% | 98% | | Hypothetical protein | |
| <i>yacA</i> | pCT_006 | → | 2629-2898 | 89 | 94% | 97% | YacA | Putative stabilisation protein | Putative homologues in IncP β plasmid B8 of toxin-antitoxin system |
| <i>yacB</i> | pCT_007 | | 2895-3176 | 93 | 96% | 97% | YacB | Putative stabilisation protein | |
| | pCT_008 | ← | 3372-3713 | 113 | 0% | 0% | | Putative membrane protein | |
| | pCT_009 | → | 4013-4600 | 195 | 0% | 0% | | Putative serine acetyltransferase | |
| <i>yafB</i> | pCT_010 | → | 4882-5475 | 197 | 95% | 95% | YafB | Metabolic proteins | |
| <i>yagA</i> | pCT_011 | → | 5829-7175 | 448 | 99% | 98% | YagA | Replication and repair protein | |
| * | pCT_012 | ← | 7710-8021 | 308 | 100% | 0% | | Hypothetical protein | |
| <i>parA like</i> | pCT_013 | ← | 8009-8635 | 209 | 100% | 0% | ParA | Putative stability protein | |
| <i>impB</i> | pCT_014 | ← | 8847-10118 | 423 | 100% | 99% | ImpB | UV protection protein | Similar arrangement to |
| <i>impA</i> | pCT_015 | ← | 10118-10555 | 145 | 100% | 99% | ImpA | UV protection protein | <i>imp</i> locus found in |
| <i>impC</i> | pCT_016 | ← | 10552-10800 | 82 | 100% | 99% | ImpC | UV protection protein | plasmid TP110 |
| | pCT_017 | → | 10904-12124 | 1406 | 0% | 0% | | Transposase | |
| <i>yccA</i> | pCT_018 | → | 12236-12505 | 89 | 100% | 100% | YccA | Inner membrane protein | |
| | pCT_019 | → | 12474-13445 | 323 | 95% | 97% | | Hypothetical protein | |
| | pCT_020 | → | 13449-13754 | 101 | 98% | 91% | | DNA methylase protein | |
| | pCT_022 | → | 14514-14735 | 73 | 99% | 90% | | Hypothetical protein | |
| * | pCT_023 | → | 14449-15183 | 144 | 95% | 92% | | Hypothetical protein | |
| <i>ycfA</i> * | pCT_024 | → | 15183-15197 | 273 | 100% | 0% | YcfA | Conserved hypothetical plasmid protein | |
| | | | 15196-15228 | | | | | | |
| | | | 15225-15863 | | | | | | |
| | | | 15866-16000 | | | | | | |

| Gene name | ID | Direction of coding | Co-ordinates | No of aa | DNA identity pO26-vir | DNA identity Collb-P9 | Protein Homolog | Function | Notes |
|--------------------|---------|---------------------|--------------|----------|-----------------------|-----------------------|-----------------|--|---|
| IS66 A | pCT_025 | → | 16037-16714 | 225 | 0% | 0% | IS66 | Transposase | Also found in plasmid pEK204 disrupting the shufflon region |
| IS66 B | pCT_026 | → | 16714-17961 | 115 | 0% | 0% | IS66 | Transposase | |
| IS66 C | pCT_027 | → | 17081-18652 | 523 | 0% | 0% | IS66 | Transposase | |
| <i>klcA</i> | pCT_028 | → | 19125-19550 | 141 | 95% | 93% | KlcA | Stable inheritance protein | |
| * | pCT_029 | → | 19597-20019 | 140 | 98% | 96% | | Hypothetical protein | Gene involved in replication |
| | pCT_030 | ← | 20071-20214 | 100 | 95% | 94% | | Hypothetical protein | |
| | | | 20214-20369 | | | | | | |
| | pCT_031 | → | 20410-20856 | 157 | 97% | 98% | | Hypothetical protein | |
| <i>ssb</i> * | pCT_032 | → | 20774-20938 | 143 | | | Ssb | ssDNA binding | Gene involved in replication |
| <i>ssb</i> | pCT_033 | → | 20977-21504 | 175 | 99% | 95% | Ssb | ssDNA binding | |
| | pCT_034 | → | 21761-21792 | 86 | 99% | 95% | YkfF | conserved hypothetical plasmid protein | |
| <i>ycjA</i> | pCT_035 | → | 21761-23812 | 654 | 94% | 93% | ParB | Partitioning protein | Gene involved in replication |
| <i>psiB</i> | pCT_036 | → | 23864-24301 | 145 | 99% | 97% | PsiB | Plasmid SOS inhibition protein | |
| <i>psiA</i> | pCT_037 | → | 24298-25017 | 239 | 99% | 97% | PsiA | Plasmid SOS inhibition protein | Gene involved in replication |
| <i>ardA</i> | pCT_038 | → | 25014-25610 | 198 | 98% | 0% | ArdA | Hypothetical protein | Gene involved in replication |
| | pCT_039 | → | 26972-26572 | 166 | 98% | 100% | | Antirestriction protein | |
| * | pCT_040 | → | 26754-26969 | 155 | 100% | 97% | YhdA CcgAll | Hypothetical protein | |
| | pCT_041 | → | 27093-27308 | 71 | 99% | 89% | | Hypothetical protein | |
| | pCT_042 | → | 27305-27739 | 144 | 99% | 98% | | Hypothetical protein | |
| <i>ygdA</i> | pCT_043 | → | 27736-28098 | 120 | 99% | 90% | | Hypothetical protein | |
| <i>ccgAll</i> | pCT_044 | → | 28200-28664 | 129 | 100% | 0% | | | |
| <i>Transposase</i> | pCT_045 | → | 28661-29551 | 296 | 98% | 89% | | Putative transposase | |
| <i>ydgA</i> | pCT_046 | → | 29551-29796 | 81 | 93% | 88% | YdhA | Putative | |
| <i>ygeA</i> | pCT_047 | ← | 29827-30078 | 83 | 98% | 98% | | Putative | |
| <i>ydHA</i> * | pCT_048 | → | 30163-30183 | 123 | 99% | 97% | | | |
| | | | 30183-30533 | | | | | | |
| <i>ydiA</i> | pCT_049 | → | 30658-31506 | 282 | 99% | 96% | YdiA | Putative | |

| Gene name | ID | Direction of coding | Co-ordinates | No of aa | DNA identity pO26-vir | DNA identity Collb-P9 | Protein Homolog | Function | Notes |
|---------------------|---------|---------------------|--------------|----------|-----------------------|-----------------------|-----------------|------------------------------|--|
| <i>yggA</i> | pCT_050 | ← | 31593-31934 | 113 | 98% | 97% | YggA | Putative | |
| <i>nikA</i> | pCT_051 | → | 32161-32493 | 110 | 100% | 71% (91%) | NikA | Relaxome component | |
| <i>nikB</i> | pCT_052 | → | 32505-35225 | 906 | 100% | 71% (91%) | NikB | Relaxase | |
| <i>trbC</i> | pCT_053 | ← | 35446-37746 | 766 | 99% | 76% (85%) | TrbC | Putative prepillin | |
| <i>trbB</i> | pCT_054 | ← | 37727-38851 | 374 | 100% | 69% (41%) | TrbB | ATPase | |
| <i>trbA</i> | pCT_055 | ← | 38848-40110 | 420 | 100% | 72% (86%) | TrbA | Regulation | |
| | pCT_056 | → | 40742-41038 | 98 | 99% | 86% | | Hypothetical protein | |
| <i>parB</i> | pCT_057 | ← | 41427-41894 | 155 | 99% | 0% | ParB | Partitioning protein | No homology to other Incl <i>parB</i> genes |
| <i>Transposase</i> | pCT_058 | ← | 42085-42840 | 251 | 0% | 0% | | Putative transposase | |
| <i>Transposase</i> | pCT_059 | ← | 42857-44392 | 511 | 0% | 0% | | Putative transposase | |
| | pCT_060 | → | 44698-45321 | 207 | 99% | 0% | | Hypothetical protein | |
| | pCT_061 | ← | 45519-45734 | 71 | 100% | 0% | | Hypothetical protein | |
| | pCT_062 | ← | 45738-46106 | 122 | 99% | 0% | | Hypothetical protein | |
| | pCT_063 | ← | 46118-46309 | 63 | 98% | 0% | | Hypothetical protein | |
| | pCT_064 | ← | 46397-46717 | 106 | 98% | 0% | | Hypothetical protein | |
| <i>pndA</i> | pCT_065 | → | 46821-47114 | 97 | 100% | 91% | PndA | Stabilisation protein | Putative post segregational killing gene |
| <i>Sigma factor</i> | pCT_066 | ← | 47259-48845 | 528 | 99% | 0% | | Putative sigma factor | Protein homology to SigB, (Sigma 70 family) found in a <i>Yersinia frederiksenii</i> (ZP_04633307) |
| <i>excA</i> | pCT_067 | ← | 49119-49772 | 215 | 100% | 68% (49%) | ExcA | Surface exclusion protein | |
| <i>traY</i> | pCT_068 | ← | 49860-52058 | 721 | 100% | 68% | TraY | Integral membrane protein | |
| <i>traX</i> | pCT_069 | ← | 52100-52669 | 189 | 100% | 72% (23%) | TraX | Pilin | |
| <i>traW</i> | pCT_070 | ← | 52666-53706 | 346 | 100% | 75% | TraW | Transfer related lipoprotein | |
| <i>traV</i> | pCT_071 | ← | 53829-54449 | 206 | 100% | 69% | TraV | | |
| <i>traU</i> | pCT_072 | ← | 54449-57493 | 1014 | 100% | 72% | TraU | Nucleotide binding protein | |
| <i>traT</i> | pCT_073 | ← | 57788-58501 | 237 | 100% | 68% (66%) | TraT | | |
| <i>traS</i> | pCT_074 | ← | 58521-58772 | 83 | 100% | 0% | TraS | | |

| Gene name | ID | Direction of coding | Co-ordinates | No of aa | DNA identity pO26-vir | DNA identity Collb-P9 | Protein Homolog | Function | Notes |
|--------------------------------|---------|---------------------|----------------------------|----------|-----------------------|-----------------------|-----------------|--|--|
| <i>traR</i> | pCT_075 | ← | 58829-59227 | 132 | 100% | 0% | TraR | | |
| <i>traQ</i> | pCT_076 | ← | 59274-59804 | 176 | 100% | 74% | TraQ | | |
| <i>traP</i> | pCT_077 | ← | 59801-60514 | 237 | 100% | 74% (22%) | TraP | | |
| <i>traO</i> | pCT_078 | ← | 60511-61848 | 445 | 100% | 71% (41%) | TraO | Pore protein | |
| <i>traN</i> | pCT_079 | ← | 61852-62826 | 324 | 100% | 75% (80%) | TraN | Secretin/pore protein | |
| <i>traM</i> | pCT_080 | ← | 62837-63532 | 231 | 100% | 73% (73%) | TraM | | |
| <i>traL</i> | pCT_081 | ← | 63544-63894 | 116 | 100% | 72% | TraL | | |
| <i>sogL</i> | pCT_082 | ← | 63911-67972 | 1353 | 100% | 74% (54%) | Sog | DNA primase | |
| | | | | | | | L/TraC | | |
| * | pCT_083 | ← | 68326-68922 72048-72083 | 232 | 0% | 0% | | Uncharacterised protein | 39.53% G/C unlike surrounding CDSs |
| IS903B | pCT_084 | ← | 68888-69370 | 322 | 0% | 0% | | Putative transposase | |
| <i>bla</i> _{CTX-M-14} | pCT_085 | ← | 69405-70280 | 291 | 0% | 0% | CTX-M-14 | β lactamase | <i>bla</i> _{CTX-M-14} 249 bp downstream (not the usual 49 bp) |
| ¹⁴ ISEcp1 | pCT_086 | ← | 70530-71792 | 420 | 0% | 0% | | Transposase | |
| <i>traK</i> | pCT_087 | ← | 72204-72494 | 96 | 100% | 69% | TraK | | |
| <i>traJ</i> | pCT_088 | ← | 72491-73639 | 382 | 100% | 75% | TraJ | Nucleotide binding protein | |
| <i>traI</i> | pCT_089 | ← | 73623-74288 | 276 | 100% | 72% (81%) | TraI | Transfer related lipoprotein | |
| <i>traH</i> | pCT_090 | ← | 74326-74913 | 195 | 100% | 67% (32%) | TraH | | |
| <i>traF</i> | pCT_091 | ← | 75017-76219 | 400 | 100% | 72% | TraF | | |
| <i>traE</i> | pCT_092 | ← | 76321-77142 | 273 | 100% | 66% (72%) | TraE | | |
| <i>Rci</i> | pCT_093 | ← | 77355-78479 | 474 | 97%(16%) | 72% (74%) | Rci | Shufflon recombinase | Although detailed in |
| <i>Shufflon A</i> | pCT_094 | → | 78534-78893 | 119 | 99%(31%) | 66% (68%) | | PilV pilus protein A | Incl plasmid R64, there is only moderate resemblance of this system to the pCT shufflon. The entire region has most DNA homology (60%) to the shufflon region of plasmid R721. |
| <i>Shufflon D</i> | pCT_095 | ← | 78898-79287 | 129 | 98% | 0% | | PilV pilus protein C' (alternative 3' tip) | |
| <i>Shufflon C'</i> | pCT_096 | → | 79298-79528 79525-79788 | 76 | 0% | 83% (98%) 79% | | PilV pilus protein C' (alternative 3' tip) | |
| <i>Shufflon C</i> | pCT_097 | ← | | 87 | 0% | (97%) | | PilV pilus protein C' (alternative 3' tip) | |

| Gene name | ID | Direction of coding | Co-ordinates | No of aa | DNA identity pO26-vir | DNA identity Collb-P9 | Protein Homolog | Function | Notes |
|-------------------|---------|---------------------|--------------|----------|-----------------------|-----------------------|-----------------|----------------------------|-------------------|
| <i>Shufflon B</i> | pCT_098 | → | 79799-80062 | 87 | 0% | 70% | | PilV pilus protein C' | <i>Shufflon B</i> |
| <i>pilV</i> | pCT_099 | ← | 80072-81337 | 421 | 99% | 73% (13%) | PilV | Type IV prepilin | |
| <i>pilU</i> | pCT_101 | ← | 81355-81981 | 208 | 99% | 0% | PilU | Prepilin peptidase | |
| <i>pilT</i> | pCT_102 | ← | 81997-82482 | 161 | 100% | 0% | PilT | Transglycosylase | |
| <i>pilS</i> | pCT_103 | ← | 82577-83063 | 178 | 100% | 0% | PilS | Type IV prepilin | |
| <i>pilR</i> | pCT_104 | ← | 83125-84219 | 364 | 99% | 0% | PilR | Pilus biogenesis | |
| <i>pilQ</i> | pCT_105 | ← | 84221-85729 | 502 | 99% | 74% (4%) | PilQ | Nucleotide binding protein | |
| <i>pilP</i> | pCT_106 | ← | 85832-86290 | 152 | 99% | 0% | PilP | Pilus biogenesis protein | |
| <i>pilO</i> | pCT_107 | ← | 86280-87575 | 431 | 99% | 0% | PilO | Pilus biogenesis protein | |
| <i>pilN</i> | pCT_108 | ← | 87596-89215 | 539 | 99% | 83% (2%) | PilN | Secretin | |
| <i>pilM</i> | pCT_109 | ← | 89247-89684 | 145 | 99% | 0% | PilM | Pilus biogenesis protein | |
| <i>pilL</i> | pCT_110 | ← | 89688-90758 | 356 | 99% | 0% | PilL | Lipoprotein | |
| | pCT_111 | ← | 90985-91341 | 122 | 100% | 0% | | Hypothetical protein | |
| <i>pilI</i> | pCT_112 | ← | 91437-91679 | 80 | 100% | 0% | PilI | | |
| <i>*</i> | pCT_113 | ← | 91760-91864 | 564 | 100% | 0% | | Hypothetical protein | |
| <i>traC</i> | pCT_114 | ← | 92170-92832 | 220 | 94% | 0% | TraC | Regulator of transfer | |
| <i>traB</i> | pCT_115 | ← | 92972-93613 | 213 | 99% | 67% (81%) | TraB | Regulator of transfer | |

Pseudogenes are denoted with *;

The percentage DNA identify to pO26_vir and Collb-P9 is followed by the percentage coverage in brackets,

aa, amino acid.

3.5.4 Antibiotic resistance and virulence genes

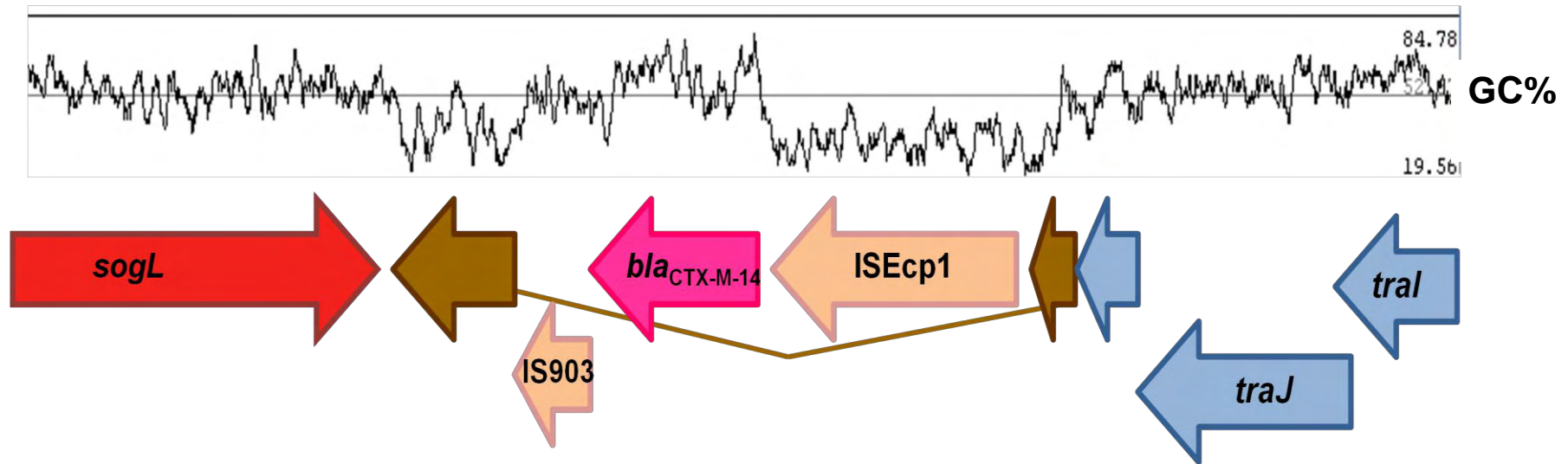
Annotation of the pCT sequence showed an absence of any genes known to contribute to virulence of the host bacterium. The only antimicrobial resistance gene identified was β -lactamase *bla*_{CTX-M-14} through which the plasmid was first identified.

3.5.4.1 *The genetic context of bla_{CTX-M-14} on pCT*

The genomic organisation of the pCT *bla*_{CTX-M-14} and flanking insertion sequences *ISEcp1* and *IS903* was found to correspond largely with other described plasmid encoded *bla*_{CTX-M-14} genes (Eckert *et al.*, 2006). *ISEcp1* (pCT_086) was found 249 bp upstream of *bla*_{CTX-M-14} on pCT, with the characteristic 42 bp directly upstream and an additional 207 bp found downstream of *ISEcp1*. *ISEcp1* had a G+C content averaging 33.95%, characteristic of a mobile genetic element (Figure 3.5). *IS903* (pCT_084) was found downstream of *bla*_{CTX-M-14} in the same position as described (Eckert *et al.*, 2006), with an average G+C content of 50.45%, more closely resembling the average for pCT as a whole.

The *ISEcp1-bla*_{CTX-M-14}-*IS903* insertional feature was positioned within a pseudogene encoding a putative protein (pCT_083). This pseudogene had a considerably lower average percentage GC content (39.53%) than both the adjacent *IS903* and surrounding upstream and downstream *tra* regions (53%) (Figure 3.5). It also appeared to be unique to pCT, as similar genes could not be found in other plasmids or in any bacterial chromosome sequences deposited in Genbank. A more general view suggests that the *ISEcp1-bla*_{CTX-M-14}-*IS903* element has inserted into the *tra* locus (next to *traL* and *traK*), which has been suggested to be a common region for genetic variation in IncI α plasmids (Leyton *et al.*, 2003).

Figure 3.5. GC % of *bla*_{CTX-M-14} gene and surrounding regions



Arrows represent pCT coding regions *bla*_{CTX-M-14} and the surrounding ORFs, indicating the size of each coding region and the direction of transcription. The GC percentage of each coding region is shown by the line graph above. A GC content over 50% is represented by a point above the straight line, and GC% is represented by a point below this line.

3.5.5 Replication genes

Replication initiator gene *repZ* and positive regulator *repY* were found at the beginning of the sequence (pCT_001 and pCT_002). This IncI group replication system relies on inhibition of pseudoknot formation providing antisense RNA-mediated inhibition, and resembles the replication system CopA/R of plasmid R100 (Kato and Mizobuchi, 1994). The same RepY/Z system can be identified in IncIα plasmid Collb-P9 (AB021078) (Asano *et al.*, 1999) with 100% DNA homology (Table 3.2). Also encoded in the pCT genome is the remnant of *repA*, an alternative replication initiator gene, which is annotated as a pseudogene and unlikely to be functional. Other pCT coding sequences (CDSs) likely to be involved in successful replication such as *ssb* (pCT_033), *ardA* (pCT_039) and *psiB/A* (pCT_036 and pCT_037) also have 98-99% identity to replication genes found in IncI plasmid Collb-P9 and R64 (AP005147); indicating the highly conserved nature of these replication related operons. The CDS encoding a DNA primase (pCT_082) has much less homology to its counterpart in plasmid Collb-P9. Protein alignments show the homolog to be either SogL in associated plasmids or TraC in IncPα type plasmids. In related plasmids such as R64, *sogL* is adjacent to *sogS*. *sogS* is believed to be the *sogL* regulator involved in suppressing *dnaG* mutations (Merryweather *et al.*, 1986); however, the R64 *sogS* appears to be absent in pCT. DNA primases are the only proteins to have been demonstrated to be transported from the donor to recipient cells during conjugation, and are believed to help generate RNA primers for DNA synthesis (Rees and Wilkins, 1989).

3.5.6 Stability and persistence genes

pCT CDSs postulated to be involved in stable inheritance of the plasmid include *yacA/B* (pCT_006 and pCT_007), *parB* (pCT_057) and *pndA* (pCT_065). pCT YacA and YacB have homologues in the IncP β plasmid B8 coding for a putative toxin/antitoxin system, however pCT seems to be missing corresponding *yacC* found in IncFII plasmids such as pHK01 (HM355591) and IncI plasmid Collb-P9.

The *parB* region of pCT encodes a ParB-'like' protein but the gene does not resemble the *parB* DNA sequences from R64 or Collb-P9. A *parA*-like (pCT_013) gene was also found elsewhere on the pCT genome but likewise shares no homology with the R64 *parA*. As these genes are unlike the fully characterised R64 *par* regions, and are not adjacent to one another, it is unclear whether they play a functional role in plasmid partitioning.

3.5.6.1 *impA/B/C UV protection or mutation repair genes*

The *imp* operon affords a mechanism to repair DNA damage resultant from UV exposure. Although the annotation and presence of an adjacent transposase suggests the UV protection gene cluster in pCT (pCT_014, pCT_015 and pCT_016) is within a transposon, this gene region is frequently found within plasmids of the IncI α complex. *impA/B* and *C* are in a similar formation to the arrangement detailed by Lodwick *et al.* (1990) in plasmid TP110 when the third component (*impC*) was first recognised and the protein products showed between 48% and 55% similarity to UmuDC and MucAB mutation repair proteins found in IncP plasmids.

3.5.7 Transfer and conjugation genes

pCT has two large conjugation systems encoded within the genome, the *tra* and *pil* operons (Figure 3.6) both controlled in part by the expression of *trbABC*.

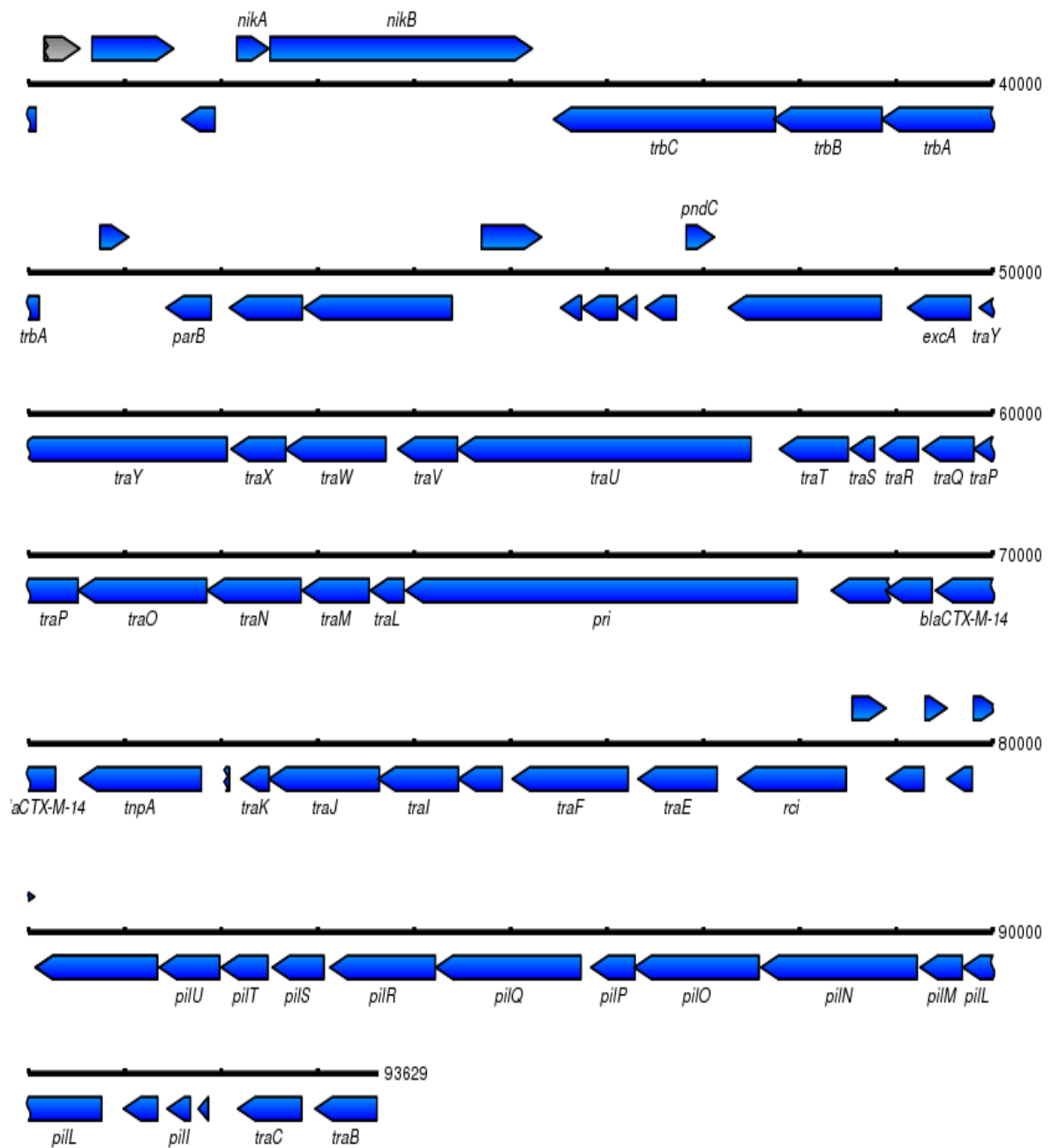
3.5.7.1 *tra* operon

The *tra* operon is believed to be essential for conjugation both in liquid and on a solid surface and encodes a thick, rigid pilus (Bradley, 1984). The *tra* genes found on pCT are separated into three regions. The first is from *traY* to *traL* where the DNA primase gene is found and the *bla*_{CTX-M-14} has inserted. The second is from *traK* to *traE* with an interruption by the shufflon and *pil* region (*rci* – *pill*); the third is made up of *traC* and *traB* at the end of the sequence (Figure 3.6). This organisation can be found in a number of other IncI complex plasmids (with the exception of the inserted *bla*_{CTX-M-14} gene). However, pCT lacks a *traA*-like region usually found next to *traB*. The annotation also indicates that the *traG* CDS is missing. On closer inspection, only 32% of the region in pCT annotated as *traH* (pCT_090) shares homology to the *traH* in plasmid Collb-P9. Although the remaining proportion of the gene has limited amino acid similarity to *traG* there are significant differences between these plasmids in this genetic region. The absence of *traG* is also found in other similar IncI complex plasmids such as pR3521 (GU256641).

3.5.7.2 *pil* operon

The second pCT transfer operon is the *pil* locus, considered a characteristic of the IncI plasmids (Bradley, 1984) it encodes a thin flexible pilus believed to increase conjugation rates in liquid. The tip of this thin pilus is variable, and believed to determine bacterial donor-recipient specificity (Komano *et al.*, 1994). The exact nature of the expressed epitope is determined by the orientation of the reversible *pilV* shufflon components (pCT_094-pCT_098), which can be inverted by the action of the recombinase Rci protein (pCT_093), encoded downstream of this region. Analysis of the pCT plasmid assembly showed that this region is actively recombining with

Figure 3.6. CDS organisation of the pCT transfer genes (*tra*, *trb* and *pil*)



The genomic organisation of the pCT transfer regions.

Putative coding regions are shown as blue arrows indicating the direction of transcription; pseudogenes are represented with grey arrows.

multiple forms of this region evident within the reads used to assemble the plasmid sequence. Although first detailed in IncI plasmid R64, there is only moderate resemblance of this recombination system to the pCT shufflon. pCT shufflon segments A, B and C had between 66% and 79% DNA identity to parts of those found in R64 (Gyohda *et al.*, 2004) but shufflon segment D (pCT_095) had no homology. The entire region had most DNA identity (60%) to the shufflon region of IncI plasmid R721 (AP002527) (Kim and Komano, 1992), which encodes an alternative shufflon operon, however this plasmid had little homology with the rest of the pCT genome (Figure 3.7).

3.5.8 Other pCT regions

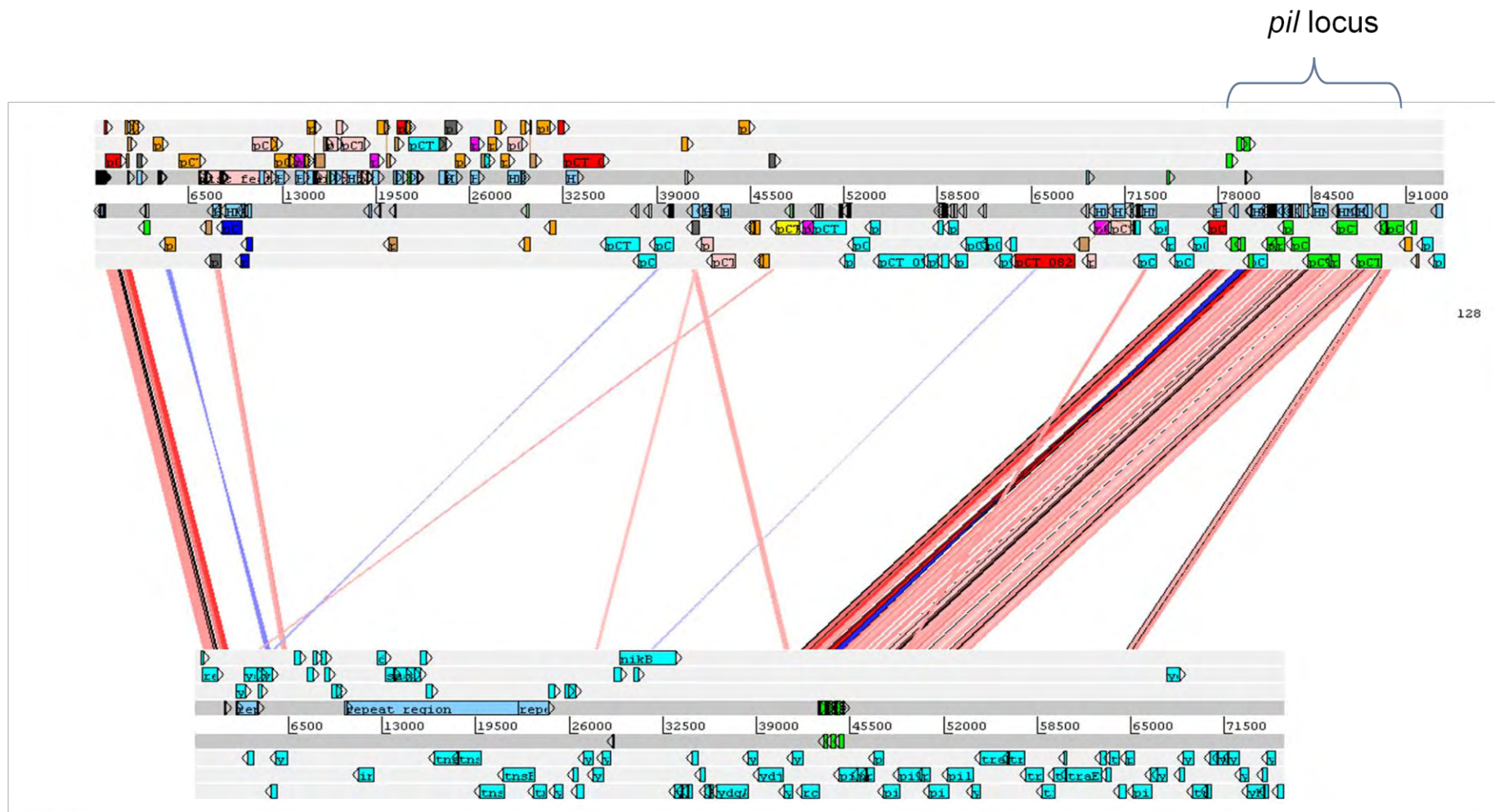
3.5.8.1 *Putative sigma factor*

The pCT CDS pCT_066 shares DNA identity with genes on five closely related plasmids (pO26vir; pR3521, pO113, TP113 and pSERB1) but is completely absent in all other plasmid sequences deposited in Genbank (As of Sept 2011). The protein encoded shows most identity to an RNA polymerase Sigma factor SigB, part of the Sigma 70 family found in a *Yersinia frederiksenii* (ZP_04633307). The presence of a sigma factor on a plasmid is unusual and may have implications for expression of genes on pCT or of its bacterial host.

3.5.8.2 *Other insertion sequences*

Insertion sequences surrounding the β -lactamase gene and genes encoding UV protection proteins were described in sections 3.3.4.1 and 3.3.6.2 however, there were other insertional elements found within the pCT sequence. Most prominent are the three CDSs which make up IS66 (pCT_025 to pCT_027), a member of the IS66

Figure 3.7. DNA sequence comparison of pCT vs. R721



Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid R721 (AP002527), represented as the bottom line of the comparison. Bands of color indicate homology between sequences. Red/Pink lines show sequence homology in the same confirmation; blue lines indicate sequence inversion. Green ORFs in pCT represent the *pil* operon (*pilA-pilV*).

family which have been shown to be mobilisable on *E. coli* plasmid pB171 (Han *et al.*, 2001). Other insertion elements include those from the IstB family, *rve* superfamily and transposase 31 super-family. However, overall, the pCT plasmid backbone encodes the relatively small number of seven transposable elements when compared to other similar large conjugative plasmids. pCT is also notably lacking IS26 which has been observed in a range of *bla*_{CTX-M} carrying plasmids on many different plasmid backbones (Eckert *et al.*, 2006).

3.5.9 Comparisons with other plasmids within the IncI complex

Although many of the genes discussed have been compared to homologous regions in other plasmids, a comparison of the whole plasmid sequence in relation to other IncI complex plasmids was beneficial when considering the evolutionary origin of pCT. Unique regions of pCT were identified for further study, and conserved regions within the plasmid group determined. For whole plasmid comparisons, comparison files of each plasmid were generated using Double ACT and then aligned using the Artemis comparison tool. BLASTn was also used to compare whole plasmid genomes and individual CDSs.

Ten complete plasmid genomes were compared to pCT using BLASTn to calculate percentage identity and coverage between each pair (Table 3.3). Those plasmids with most homology to pCT were pO26_vir (FJ38659) (Figure 3.8), pR3521 (Figure 3.9), pO113 (AY258503) (Figure 3.10) and the partial sequence of pSERB1 (AY686591); none of which carry *bla*_{CTX-M} genes. The greatest homology was found between pCT and pO26_vir. 85% of the pCT genome had identity with genes found on pO26_vir including the putative sigma factor, the majority of the *pil* locus and all *tra* genes (Table 3.2). However, pO26_vir is a large plasmid of 168,100 bp

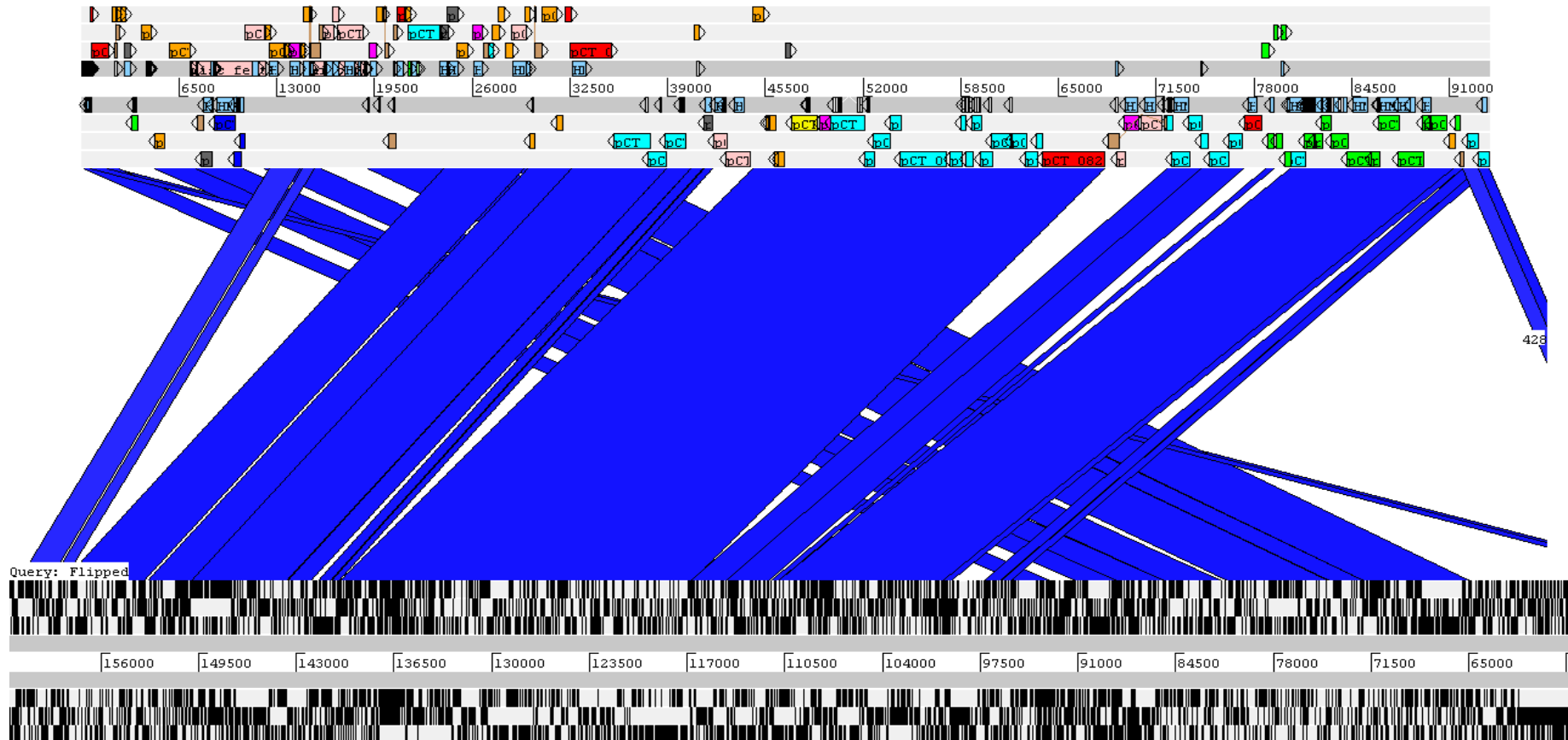
Table 3.3. pCT complete sequence homology across whole genomes of available sequenced plasmids

| Plasmid | Inc type | Organism | Sorce | Coverage of pCT | % homology | Accession no. | Reference/ date deposited |
|----------|----------|--------------------------|-------------|-----------------|------------|---------------|---|
| pO26-vir | B | <i>E. coli</i> | Unspecified | 85% | 99% | FJ386569 | 31 st March 2009 |
| pR3521 | B | <i>E. coli</i> | Human | 83% | 100% | GU256641 | Papagiannitsis <i>et al.</i> , 2011 |
| pO113 | Unknown | <i>E. coli</i> | Human | 82% | 100% | AY258503 | Srimanote <i>et al.</i> , 2002 Leyton <i>et al.</i> , 2003 |
| TP113 | B | <i>E. coli</i> | Unspecified | 83% | 99% | Not deposited | Grindley <i>et al.</i> , 1972 |
| R387 | K | <i>Shigella flexneri</i> | Unspecified | 70% | 99% | Not deposited | Shaw <i>et al.</i> , 1972 |
| pSERB1 * | K | <i>E. coli</i> | Human | 66% | 99% | AY686591 | Dudley <i>et al.</i> , 2006 |
| pECOED | Unknown | <i>E. coli</i> | Unspecified | 62% | 99% | CU928147 | 16 th December 2008 |
| pEK204 | I1α | <i>E. coli</i> EO516 | Human | 60% | 100% | EU935740 | Woodford <i>et al.</i> , 2009 |
| Collb-P9 | I1α | <i>Shigella sonnei</i> | Unspecified | 54% | 99% | AB021078 | Mankovich <i>et al.</i> , 1986 |
| R64 | I1α | <i>S. Typhimurium</i> | Human | 54% | 99% | AP005147 | Meynell and Datta, 1966 Sampei <i>et al.</i> , 2010 |

*Incomplete sequence,

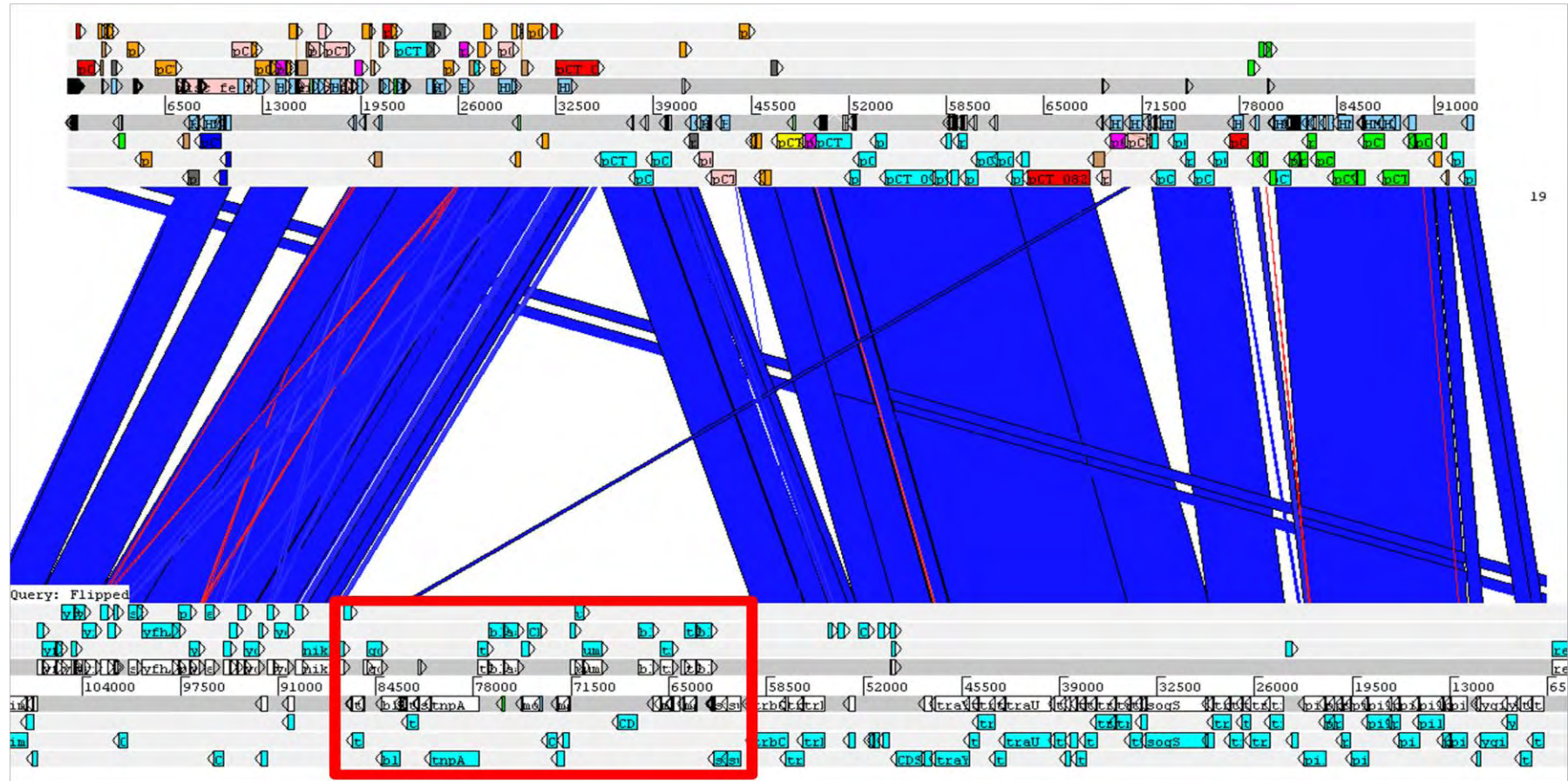
Plasmids included are those available in Genbank as of Septeber 2011.

Figure 3.8. DNA sequence comparison of pCT vs. pO26_vir



Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid pO26_vir (FJ386569), represented as the bottom line of the comparison. Bands of color indicate homology between sequences, blue lines indicate sequence inversion. As pO26_vir has not been fully annotated and the coding regions not fully defined, the pO26_vir stop and start codes are indicated instead, represented as black lines.

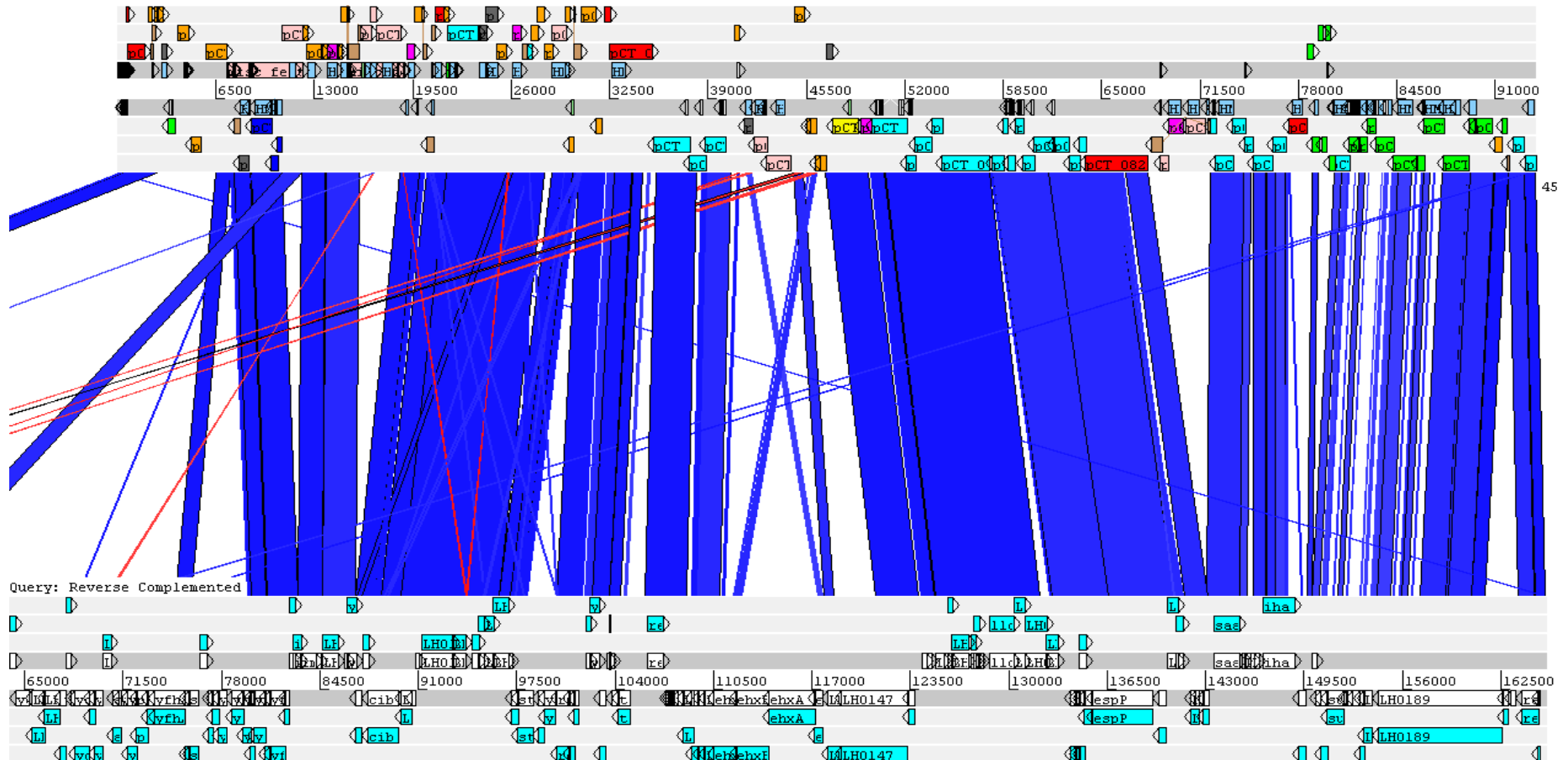
Figure 3.9. DNA sequence comparison of pCT vs. pR3521



Resistance cassette

Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid pR3521 (GU256641), represented as the bottom line of the comparison. Bands of color indicate homology between sequences. Red/Pink lines show sequence homology in the same confirmation; blue lines indicate sequence inversion. The pR3521 resistance cassette which encodes seven antibiotic resistance genes ((26,382 bp) and is absent in the pCT sequence, is indicated with a red box.

Figure 3.10. DNA sequence comparison of pCT vs. pO113



Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid pO113 (AY258503), represented as the bottom line of the comparison. Bands of color indicate homology between sequences. Red/Pink lines show sequence homology in the same confirmation; blue lines indicate sequence inversion.

encoding at least seven virulence genes not found in pCT. Areas of pCT absent in pO26_vir included the *bla*_{CTX-M-14} gene and flanking sequences, parts of the shufflon region and the shufflon recombinase and other insertion sequences.

Plasmid pR3521 (110,416 bp) (Papagiannitsis *et al.*, 2011) had less total homology with 83% coverage of the pCT genome at 100% similarity. Differences include the absence of a shufflon recombinase, where in pR3521 an unrelated hypothetical protein can be found downstream of *pilV* instead; the presence of two CDSs encoding putative adhesive threads (*yqiJ/K*) previously identified in pathogenic *E. coli* (Antao *et al.*, 2009); and the presence of a 26,382 bp multidrug resistance region. This cassette appears to have inserted into an ORF which can also be found intact on closely related plasmid pO113, further suggesting a recent mobilisation. The MDR segment of pR3521 encodes seven antibiotic resistance genes *sul1*, *strA* and *strB*, *aacC2*, *bla*_{SCO-1} (class A beta-lactamase), *bla*_{ACC-4} (*Hafnia* derived ESBL) and two copies of *bla*_{TEM-1b}.

Plasmid pO113 (165,548 bp), a very similar plasmid to pR3521, also shares key features with pCT including the *pil* locus (but the shufflon recombinase is absent), the putative sigma factor and many transfer genes (Figure 3.8). pSERB1 has 99% homology to 66% of the pCT genome, however is only deposited in Genbank as a partial sequence therefore the total identity with pCT cannot be assessed.

When considering which regions of pCT are conserved among the IncI complex plasmids it is of benefit to compare the pCT genome to other reference plasmids within this group. The best characterised IncI reference plasmid is R64, a conjugative plasmid originally isolated from an *S. Typhimurium* (Table 3.3). 64% of the pCT genome was found to have 99% homology with the genome of plasmid R64,

primarily with genes involved in replication and conjugation. Although R64 has a functional shufflon, when compared to the shufflon and *pil* locus found in pCT, these regions have ~59% DNA homology. R64 does not encode the *bla*_{CTX-M-14} gene although does encode genes which confer resistance to arsenic, tetracycline and streptomycin, which are absent in pCT (Figure 3.11).

Collb-P9 (Table 3.2) is a similar plasmid to R64 and shares the same percentage identity to pCT. Other reference plasmids compared included those used by the Sanger Institute as examples of different incompatibility types. R387 is the IncK reference plasmid (Figure 3.12) and while many regions of R387 share high percentage identity to pCT, such as the *pil* and *tra* loci, many regions of pCT are completely absent in R387, including the shufflon region, UV protection locus and the sigma factor. TP113 is the Sanger reference plasmid for the IncB group of plasmids (this group is also part of the larger IncI complex) and when this plasmid was compared to pCT (Figure 3.13) there was more sequence identity between pCT and TP113 than between pCT and R387. Those regions in pCT absent in TP113 are the *bla*_{CTX-M-14} gene, the shufflon region and recombinase and other insertion sequences.

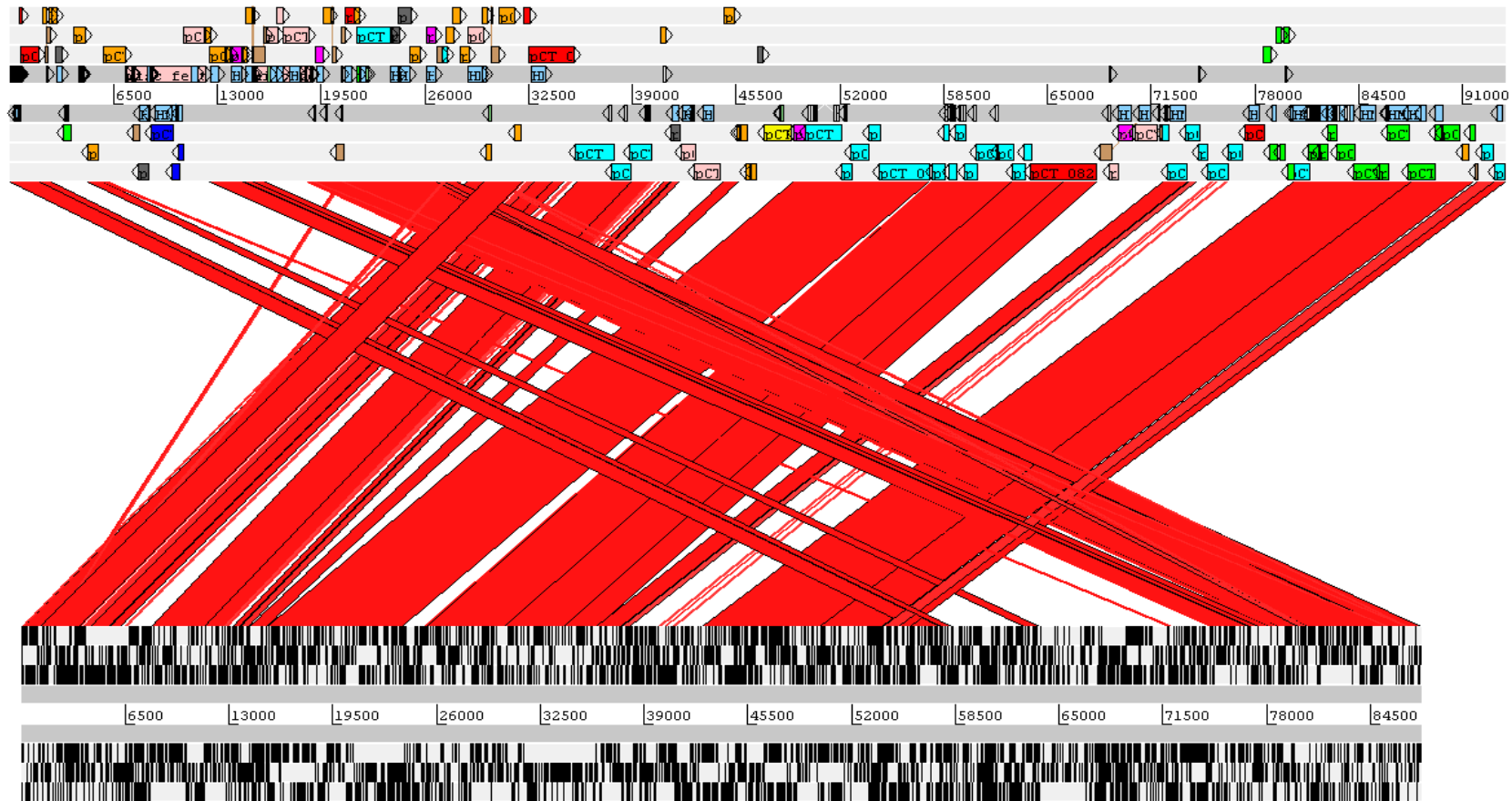
3.5.10 Comparisons of pCT with other *bla*_{CTX-M} encoding plasmids

Within the literature certain *bla*_{CTX-M} gene variants have been considered to be associated with 'epidemic' plasmids or particular plasmid incompatibility groups (Baudry *et al.*, 2009). *bla*_{CTX-M-15} is currently the most frequently detected *bla*_{CTX-M} worldwide and is most commonly found on IncFII plasmids. Similarly, both *bla*_{CTX-M-9} and *bla*_{CTX-M-32} are suggested to be carried on epidemic plasmids (Cantón and Coque, 2006). To identify any common features which may be related to the evolutionary success of these plasmids or the acquisition of the *bla*_{CTX-M} resistance



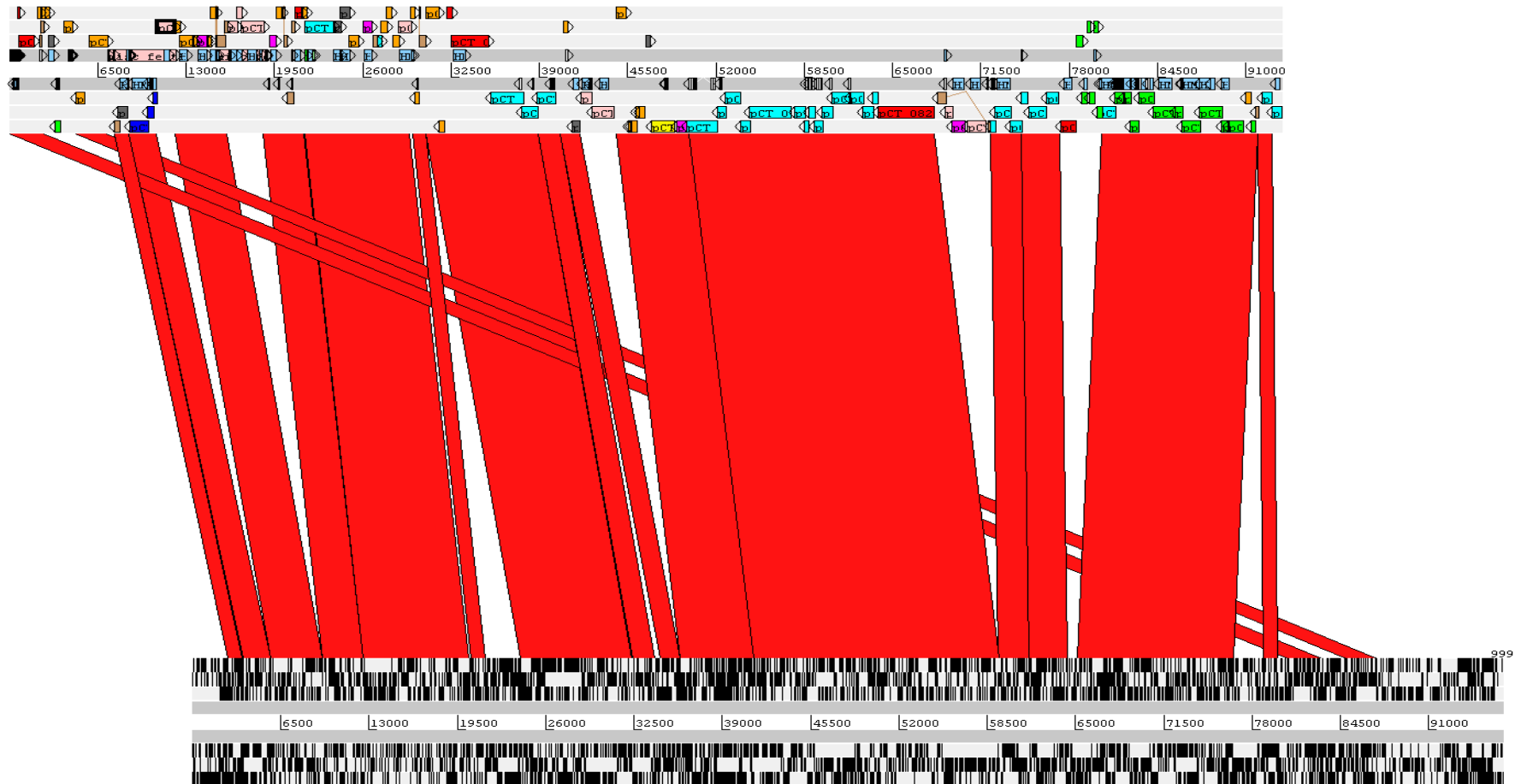
110

Figure 3.12. DNA sequence comparison of pCT vs. R387



Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid R387 (Shaw *et al.*, 1972), represented as the bottom line of the comparison. Bands of color indicate homology between sequences. Red/Pink lines show sequence homology in the same confirmation; blue lines indicate sequence inversion.

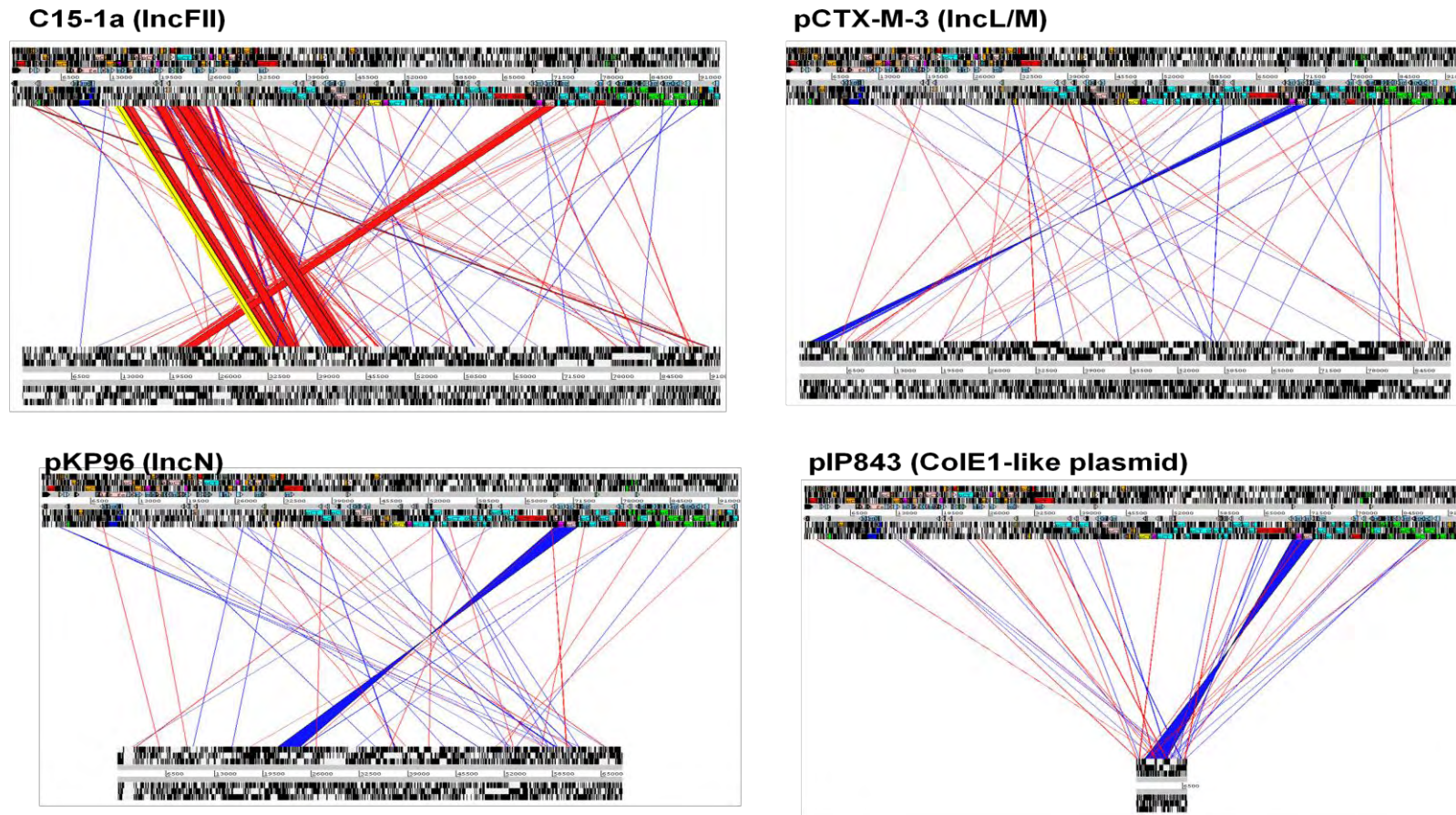
Figure 3.13. DNA sequence comparison of pCT vs. TP113



Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid TP133 (Grindley *et al.*, 1972), represented as the bottom line of the comparison. Bands of color indicate homology between sequences. Red/Pink lines show sequence homology in the same confirmation; blue lines indicate sequence inversion.

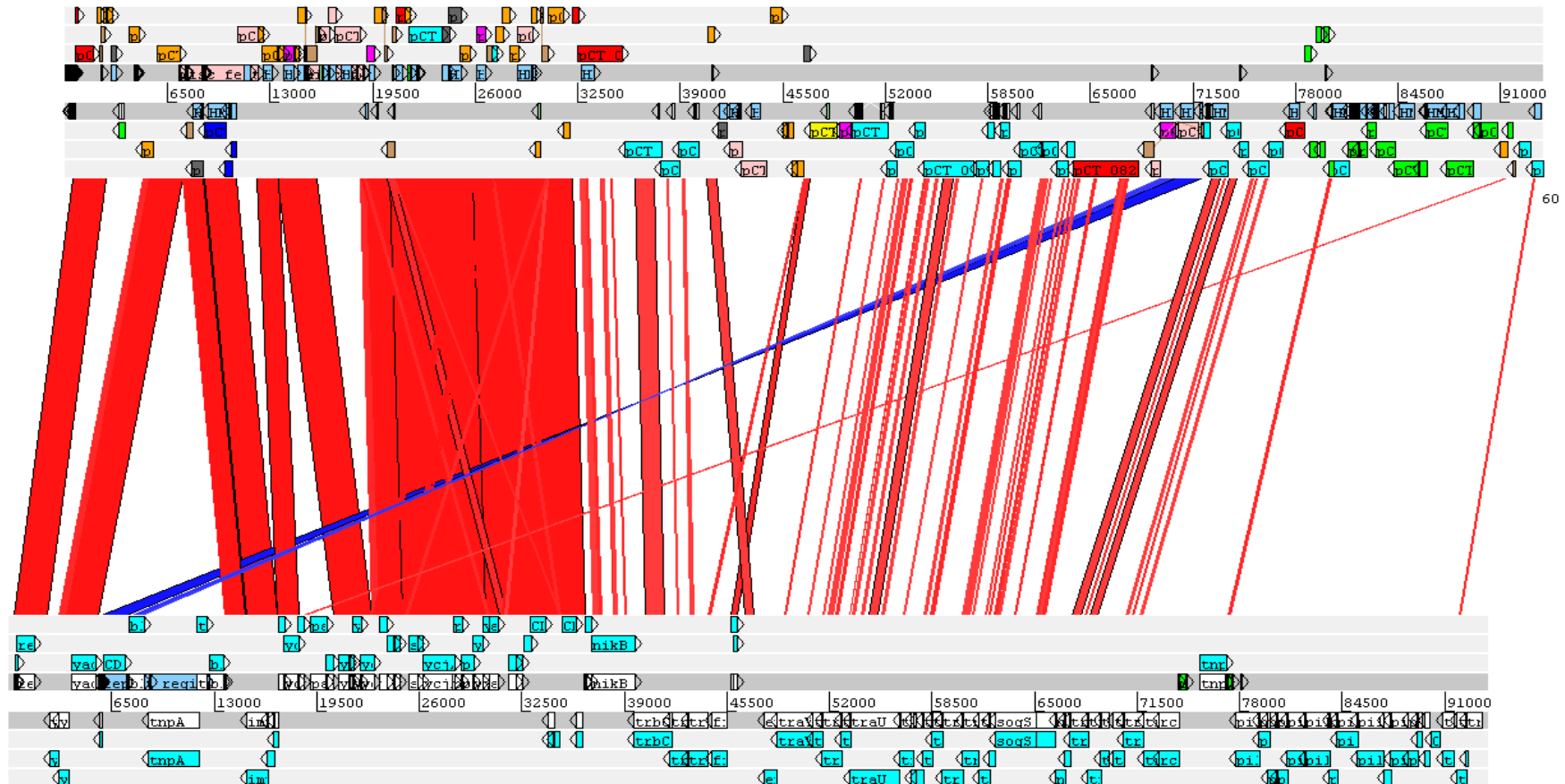
genes, the sequence of pCT was compared to other *bla*_{CTX-M} carrying plasmids. Complete sequences deposited in Genbank of plasmids carrying *bla*_{CTX-M} genes were acquired and compared with pCT using ACT (Figure 3.14). Those plasmids with different replication mechanisms such as IncFII plasmids described by Woodford *et al.*, (2009) or IncN plasmid pKP96 (Shen *et al.*, 2008) have almost no DNA homology to pCT outside the *bla*_{CTX-M} (Figure 3.14). The only other *bla*_{CTX-M} carrying IncI complex plasmid to be sequenced and deposited in Genbank thus far is a *bla*_{CTX-M-3} carrying IncI plasmid pEK204 (EU935740). pEK204 shares homology to approximately 60% of the pCT genome (Figure 3.15, Table 3.3); most of which is over IncI related core genes for replication or transfer. Further similarities between pEK204 and pCT were the minimal carriage of resistance genes (*bla*_{CTX-M-3} and *bla*_{TEM-1}) and the presence of the shufflon/*pil* operon in both plasmids. In pEK204 the shufflon region appears to have been disrupted by insertion sequences IS66 (also found within the pCT genome in a different location); therefore it is postulated to be non-functional in pEK204. As of Sept 2011, three other complete plasmid sequences which encode *bla*_{CTX-M-14} had been deposited in Genbank; all belonged to the incompatibility group IncFII. pCT and pHK01 (70262 bp; HM355591) (Ho *et al.*, 2011) shared very little in common with ~18% similarity in a few regulatory genes such as *psiB* and *ssb*. The same is true of pKF3-70 (70,057bp, FJ494913.1) (Yi *et al.*, 2010) and pTN48 (165,657 bp, FQ482074) (Billard-Pomares *et al.*, 2011), a much larger plasmid than pCT which carries numerous antibiotic resistance genes, virulence genes and multiple addiction systems. All three plasmids also carried the *bla*_{CTX-M-14} related insertion sequences *ISEcp1* and *IS903* as in pCT, however, did not have adjacent ORFs similar to pCT (e.g. *traK*, and the pCT pseudogene).

Figure 3.14. DNA sequence comparisons of pCT vs. other *bla*_{CTX-M} carrying plasmids



Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid C15-1a (NC_005327), pCTX-M-3 (AF550415), pKP96 (EU195449) and pIP843 (AY033516), represented as the bottom line of the comparison. Bands of color indicate homology between sequences. Red/Pink lines show sequence homology in the same confirmation; blue lines indicate sequence inversion.

Figure 3.15. DNA sequence comparison of pCT vs. pEK204



Artemis Comparison Tool (Sanger, UK) was used to compare the complete DNA sequence of pCT, represented as the top line of the comparison, with the complete sequence of plasmid pEK204 (EU935740), represented as the bottom line of the comparison. Bands of color indicate homology between sequences. Red/Pink lines show sequence homology in the same confirmation; blue lines indicate sequence inversion. *bla* genes on both plasmids are connected by the blue line indicating an inversion of this gene.

3.6 New diagnostic tool to detect plasmid pCT-like plasmids

3.6.1 Design of a specific PCR assay for detection of the pCT backbone

The completion of the pCT sequence annotation and subsequent comparison of the pCT genome to other IncI sequenced plasmids allowed insight into which pCT genomic regions are conserved and common, and those which were less well characterised or unique. Five genes in addition to the *bla*_{CTX-M-14} were chosen to form the basis of the PCR assay, these included the combination of universal IncI genes and more unusual sequences, encoded in different locations on the plasmid. *bla*_{CTX-M-14} was chosen as a positive control, as it was the spread of this gene which was of primary interest (primer no. 819/820). The relaxase gene *nikB* (pCT_052) was selected as a universal coding region for amplification, as work described by Francia *et al.* (2004), and Garcillan-Barcia *et al.* (2009) showed conservation of this gene between IncI group plasmids, thus allowing detection of all IncI plasmids using one set of primers. Despite an available sequence for primers designed to *nikB*, alignments with pCT and other *nikB* sequences suggested a better pairing elsewhere within the sequence. Therefore these alternative primers were used in this study (Table 2.6, primer no. 979/980). The shufflon recombinase gene (*rci*) (pCT_093) was selected for amplification as this gene is only found in plasmids which encode a *pil* locus and corresponding shufflon region, further discriminating between the IncI plasmids (primers no. 875/876). The genes encoding the putative sigma factor (pCT_066) and *pilN* (pCT_108) were chosen as they were only found in a few closely related plasmids to pCT such as p026_vir but are absent in most other characterised IncI and IncK type plasmids (primers no. 868/869 and 916/917). The last region chosen for amplification was a unique intergenic region not seen in any other plasmid

between CDSs encoding a putative membrane protein and putative serine acetyltransferase (pCT_008-pCT_009)(primer no. 983/984). Sequences were also selected to ensure a distribution of target regions for amplification around the pCT genome.

All five PCR assays were initially optimised independently for the annealing temperatures and extension times using pCT DNA isolated from DH5 α pCT (I755) as a template. Once optimised, PCR assays were combined to establish which assays could be carried out as a multiplex in the same reaction. Successful amplification of the sigma factor gene, *pilN*, and *rci* was achieved at an annealing temperature of 53⁰C in one reaction. As both *bla*_{CTX-M} group 9 and *nikB* gene PCR amplimers were used for DNA sequencing, these PCR assays remained as monoplexes. The PCR assay to amplify pCT008-pCT009 also remained as a monoplex as this region could not be amplified when placed with any other reactions. However, the successful combination of three PCRs reduced the detection of pCT-like plasmids to four simple reactions including the positive control.

3.6.2 *bla*_{CTX-M-14} and flanking regions

To establish whether the insertion site of *ISEcp1-bla*_{CTX-M-14}-*IS903* was conserved in other plasmids, two PCR assays were designed to each end of this insertion element (Figure 2.1). Primers were designed to amplify regions from *ISEcp1* to external gene *traK* (pCT_087) and from *bla*_{CTX-M-14} to the adjacent pseudogene (pCT_083) and reactions optimised using pCT DNA from a bacterial lysate.

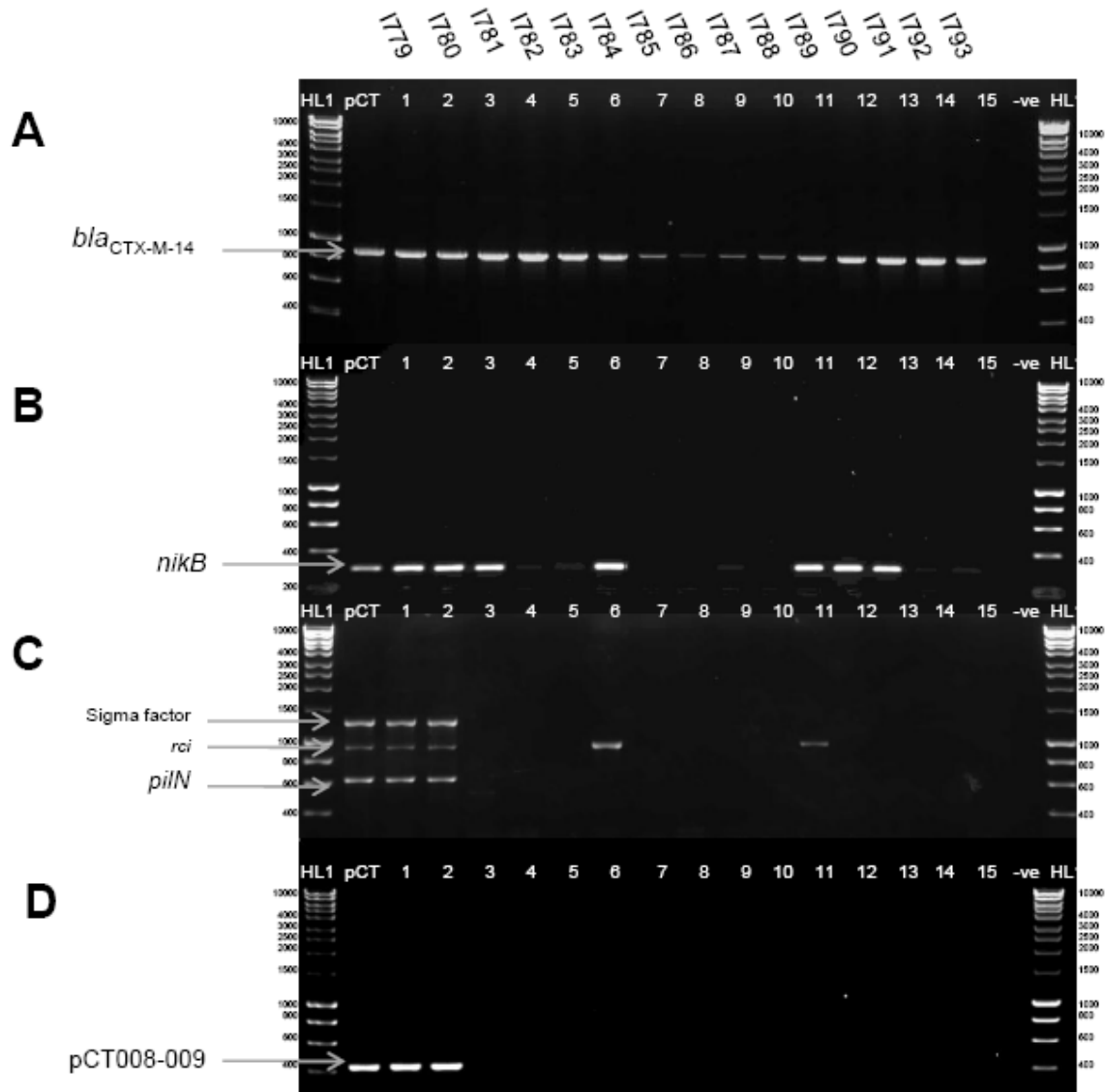
A pCT-like plasmid was defined as a plasmid from which an amplimer of the expected size was generated in all of the six PCR reactions and shared the same *bla*_{CTX-M-14} insertion site.

3.6.3 Validation of the PCR assay to detect pCT-like plasmids

In order to validate the PCR assay for the detection of pCT-like plasmids, the assay was tested on fifteen lysates made from CTX-M-14 producing *E. coli* isolates, collected from healthy cattle on ten farms distributed across England and Wales (Table 2.3, I779-I793), and obtained from collaborators at the Veterinary laboratories Agency (VLA). Plasmids within each isolate had been previously characterised for incompatibility type, however this data was kept from the investigator at the time of testing to prevent bias. Each of the fifteen lysates yielded an amplicon in the control PCR for *bla*_{CTX-M-14}, checked by sequencing to ensure the *bla*_{CTX-M} type (Figure 3.16A), 8/15 encoded a *nikB* sequence (Figure 3.16B) and 4/15 produced an *rci* amplicon (Figure 3.14C). Two isolates (I779 and I780) yielded amplicons for all PCR assays therefore, were deemed pCT-like (Figure 3.17). These results were subsequently found to be consistent with the Inc type of each plasmid.

Other isolates which contained IncI type plasmids gave amplicons for *nikB* and/or *rci* but not the more specific PCRs. The IncFII plasmid from isolate I784 did not yield an amplicon for any assay except for *bla*_{CTX-M-14}, demonstrating the specificity of this screening tool (Table 3.4). Of those isolates identified as carrying pCT plasmids, the first (I779) was later revealed to be from the same farm in North West Wales that *E. coli* C159/11 and pCT originated, but two years later in 2006 and in a different *E. coli* host sequence type. The second (I780) was isolated in Lancashire in 2008 from a farm with no direct known link to the Welsh farm, indicating the spread of this plasmid in bacteria from both Welsh and English cattle.

Figure 3.16. PCR detection of pCT-like plasmids in veterinary isolates



PCR assays used DNA lysates of *E. coli* DH5 α pCT as a positive control, 1-15, CTX-M-14 producing *E. coli* isolates collected from cattle around England and Wales (I779-I793) and *E. coli* DH5 α as a negative control (-ve).

The PCR reactions were as follows:

A, Detection of group 9 *bla*_{CTX-M} genes;

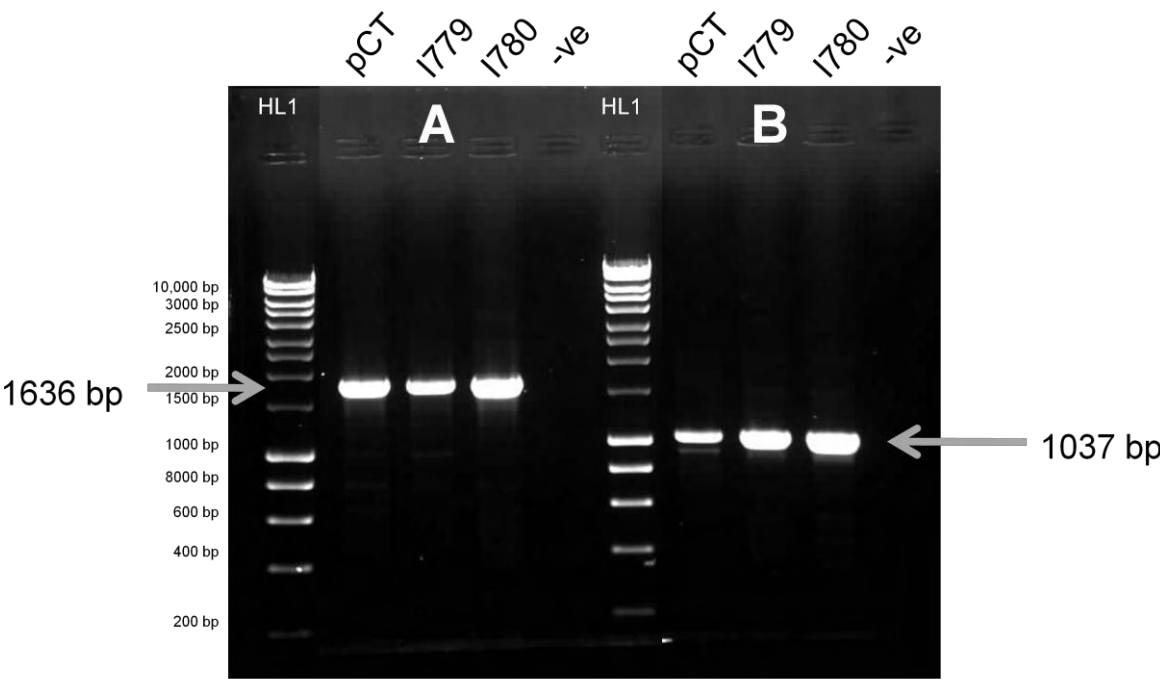
B, Detection of IncI group relaxase gene *nikB*;

C, Detection of IncI gene *rci* shufflon recombinase, the pCT putative sigma factor and *pilN* locus secretin *pilN*;

D, Detection of the pCT specific region pCT008-009.

DNA amplimers were visualised using electrophoresis on a 1% agarose gel.

Figure 3.17. Amplification of the *bla*_{CTX-M-14} insertion region *traK* to *ISEcp1* and *bla*_{CTX-M-14} to the adjacent pseudogene



| Feature | Description |
|---------|---|
| HL1 | Hyperladder 1 (Bioline) |
| A | <i>bla</i> _{CTX-M-14} to the adjacent pseudogene (1636 bp) |
| B | <i>ISEcp1</i> to <i>traK</i> (1037 bp) |
| pCT | pCT plasmid DNA from a DH5α pCT |
| I779 | Plasmid DNA from veterinary <i>E. coli</i> isolate I779 |
| I780 | Plasmid DNA from veterinary <i>E. coli</i> isolate I780 |

PCR used DNA lysates of all positive control *E. coli* DH5α pCT and test isolates *E. coli* I779 and *E. coli* I780. A lysates of *E. coli* DH5α was used as a negative control.

DNA amplimers were visualised using electrophoresis on a 1% agarose gel

Table 3.4. Summary of PCR assays for the detection of pCT-like plasmids

| Strain/ Plasmid | Inc type | Amplicers generated from PCR assays | | | | | | |
|--------------------|-------------|-------------------------------------|-------------|------------|-------------|-----------------|-------------------|--|
| | | <i>bla</i> _{CTX-M-14} | <i>nikB</i> | <i>rci</i> | <i>pilN</i> | Sigma Factor | pCT008 -pCT009 | <i>traK-ISEcp1</i> <i>bla</i> -pseudogene |
| pCT | K | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| I779 | K/F | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| I780 | K/F | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| I781 | FIA | ✓ | ✓ | x | x | x | x | x |
| I782 | F | ✓ | ✓ | x | x | x | x | x |
| I783 | - | ✓ | ✓ | x | x | x | x | x |
| I784 | - | ✓ | ✓ | ✓ | x | x | x | x |
| I785 | - | ✓ | x | x | x | x | x | x |
| I786 | I1y | ✓ | ✓ | x | x | x | x | x |
| I787 | - | ✓ | ✓ | x | x | x | x | x |
| I788 | - | ✓ | ✓ | x | x | x | x | x |
| I789 | - | ✓ | ✓ | ✓ | x | x | x | x |
| I790 | - | ✓ | ✓ | x | x | x | x | x |
| I791 | F | ✓ | ✓ | x | x | x | x | x |
| I792 | F | ✓ | ✓ | x | x | x | x | x |
| I793 | F | ✓ | ✓ | x | x | x | x | x |
| L125 | - | ✓ | x | x | x | x | x | x |
| 386 | FII | ✓ | x | x | x | x | x | x |
| 400 | FII | ✓ | x | x | x | x | x | x |
| C574 | K | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| C559 | K | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| C567 | K | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| pRYC105 | K | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| pRYC110 | HI2 | ✓ | x | x | x | x | x | x |
| pOZ174 | - | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| JIE 052 | B | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| JIE 081 | FII | ✓ | x | x | x | x | x | x |
| JIE 084 | FII | ✓ | x | x | x | x | x | x |
| JIE 088 | I1 | ✓ | ✓ | x | x | x | x | x |
| JIE 182 | B | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |
| JIE 201 | K | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ | ✓ |

Ticks represent a PCR assay yielding the expected sized amplicer. Shaded rows show isolates which have yielded amplicers in all PCR assays, indicating the presence of a pCT-like plasmid. Dashes, unknown Inc type.

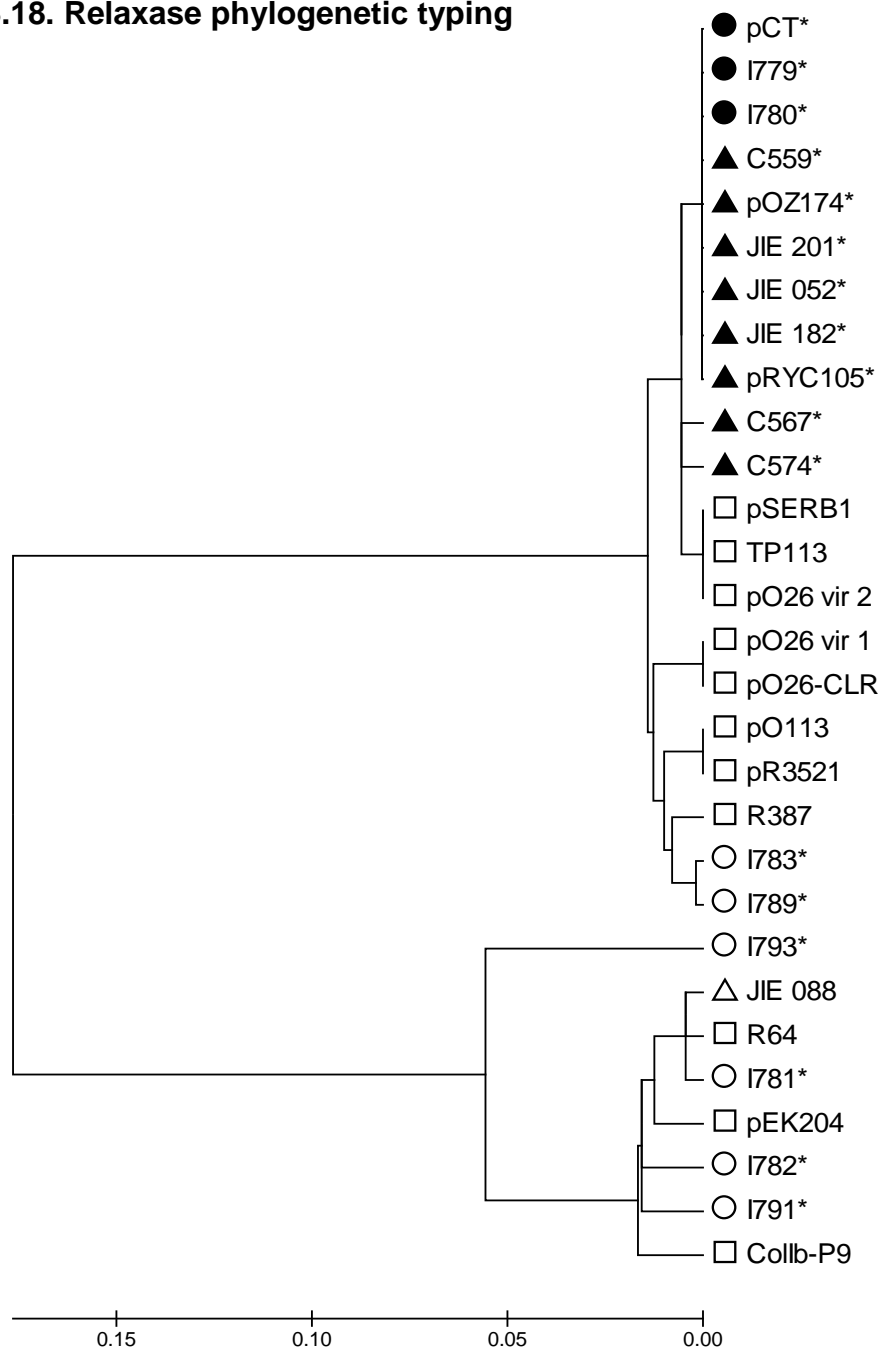
3.7 pCT global epidemiology

Previously published CTX-M-14 producing clinical isolates of *E. coli* were requested from twenty research groups globally (Table 2.3). On receipt of each isolate received, strains were cultured on LB agar containing 8 µg/ml of cefotaxime, checked for the presence of a *bla*_{CTX-M-14} using PCR and sequencing, and confirmed to be an *E. coli* by Gram stain, API and culture on XLD agar. Fifteen CTX-M-14 producing *E. coli* clinical isolates from England, Germany (Cullik *et al.*, 2010), Spain (Vinue *et al.*, 2008; Valverde *et al.*, 2009), Australia (Zong *et al.*, 2008) and China (Chanawong *et al.*, 2002) were acquired and examined for the presence of pCT using PCR assays. PCR did not detect pCT-like plasmids in isolates from England or Germany. However, 4/5 clinical isolates from Spain (C559;C567;C574;FEC383), 3/6 isolates from Australia (JIE052; JIE182; JIE201) and the isolate from China (*E. coli* 8-CH13) contained pCT-like plasmids; amplifying the expected sized fragment in each of the PCR assays (Table 3.4). Each of the eight pCT-like plasmids also had identical *bla*_{CTX-M-14} insertion regions (*bla*_{CTX-M-14} to the pseudogene and *ISEcp1* to *traK*).

3.7.1 Phylogenetic analysis using *nikB* sequences

Amplicons generated by PCR amplification of *nikB* revealed all ten of the pCT-like plasmids had *nikB* sequences with >98% DNA identity to pCT. These sequences, along with other IncI *nikB* sequences collected from Genbank were used to construct a *nikB* phylogenetic tree. pCT-like plasmids were found to cluster together with pCT regardless of origin, while the non-pCT-like plasmids clustered further away (Figure 3.18).

Figure 3.18. Relaxase phylogenetic typing



Phylogenetic analysis of *nikB* in IncI complex plasmids. DNA sequences of *nikB* PCR amplicons and sequences obtained from public resources were aligned and analysed using MEGA 4.0. A neighbor-joining tree was constructed using the maximum composite likelihood method and was linearised. Circles, *nikB* sequences from plasmids isolated from *E. coli* veterinary isolates from the United Kingdom; triangles, *nikB* sequences of plasmids from *E. coli* isolated from humans; squares, *nikB* sequences of plasmids obtained from GenBank/Sanger Institute; shaded shapes, plasmids identified as pCT-like by using PCR in this study; asterisks, plasmids encoding *bla*_{CTX-M-14}. The scale bar indicates nucleotide substitutions per site.

3.7.2 Further characterisation of the pCT-like plasmids

In order to further compare the ten identified pCT-like plasmids to pCT, each was extracted from the original *E. coli* host and transformed into *E. coli* DH5 α electro-competent cells by selecting on agar containing 8 μ g/ml of cefotaxime (Appendix 2). All ten pCT-like plasmids conferred resistance to β -lactams only, and were not found to confer resistance to ciprofloxacin, kanamycin, gentamicin, erythromycin, tetracycline, chloramphenicol or faropenem (Table 3.5). This suggests that these plasmids do not encode any other commonly plasmid mediated resistance genes (other than *bla*_{CTX-M-14}) and that, as in pCT, *bla*_{CTX-M-14} is likely to be the only antibiotic resistance gene encoded. All pCT-like plasmids were also able to conjugate from donor DH5 α to recipient DH5 α rif^R (Table 3.5).

3.8 Discussion

Plasmid pCT isolated from veterinary *E. coli* strain C159/11 was successfully transformed into *E. coli* DH5 α and transferred to *E. coli* J53-2, *E. coli* 3950 (C159/11 pCT cured strain) and *S. Typhimurium* SL1344 rif^R by conjugation. The only antibiotic resistance phenotype found to be transferrable by both transformation and conjugation of pCT was resistance to β -lactam antibiotics. This indicates that genes responsible for C159/11 resistance to the other classes of antibiotic are either chromosomally encoded or carried on other plasmids, and that *bla*_{CTX-M-14} was likely to be the only resistance gene carried on plasmid pCT. This was confirmed by sequencing of the pCT genome, however contradicts the previous publication on this plasmid which suggested that pCT also encoded a streptomycin resistance gene. A lack of prior knowledge of the *S. Typhimurium* strain used as a recipient for conjugation by Liebana *et al.* (2006) is likely to have led to this error.

Table 3.5. Further characterisation of pCT-like plasmids, the ability to conjugate and transferable resistance profiles

| Lab code | Plasmid | Conjugation ^a | Minimum Inhibitory Concentration (µg/ml) | | | | | | | | |
|----------|---------------------------------|--------------------------|--|------------|--------|-----|------|-----|-----|-----|------|
| | | | CTX | AMP | CIP | KAN | GEN | ERY | TET | CHL | FAR |
| I825 | Host strain <i>E. coli</i> DH5α | | 0.03 | 2 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.25 |
| I755 | pCT | ✓ | 16 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.5 |
| I779 | pI779 | ✓ | 16 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.5 |
| I780 | pI780 | ✓ | 8 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.5 |
| I801 | pC559 | ✓ | 8 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.5 |
| I802 | pC567 | ✓ | 16 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.5 |
| I803 | pC574 | ✓ | 16 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.25 |
| I853 | pRYC105 | ✓ | 32 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.25 |
| I832 | pOZ174 | ✓ | 16 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.25 |
| I849 | pJIE052 | ✓ | 8 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.25 |
| I850 | pJIE201 | ✓ | 8 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.25 |
| I865 | pJIE182 | ✓ | 8 | 256 | 0.0015 | 0.5 | 0.12 | 32 | 1 | 1 | 0.25 |

CTX, cefotaxime; AMP, ampicillin; CIP, ciprofloxacin; KAN, kanamycin; GEN, gentamicin; ERY, erythromycin; TET, tetracycline; CHL, chloramphenicol; FAR, faropenem. Bold type MIC data, resistance conferred by the plasmid. α, from *E. coli* DH5α to *E. coli* DH5α rif^R.

Through complete sequencing, pCT was shown to be a 93,629 bp plasmid, harbouring 115 putative open reading frames. Replication genes identified within the sequence agreed with the previous assessment detailed by Liebana *et al.*, (2006) that pCT is an IncK plasmid within the IncI complex group. The plasmid backbone itself may at first glance be regarded as unremarkable as no known virulence genes were identified and no additional antimicrobial resistance genes detected. The majority of the putative coding CDSs were as typical for this class of plasmid backbone. The UV protection operon, while possibly located within a transposon, is found on a variety of IncI plasmids and has probably been situated on these plasmids for some time. Similarly, the entire *tra* locus appeared to be conserved within this group of plasmids with minimal differences in ORFs found on pCT when compared to reference plasmids (Table 3.1).

The *pil* locus and shufflon region are also characteristic for the IncI group (Bradley, 1980), however on closer inspection this region on pCT shares only moderate identity to the corresponding locus on reference plasmid R64, where the *pil* locus and corresponding shufflon was first detailed (Komano *et al.*, 2000). The uncharacterised shufflon second segment has no known homologue, and segment D can only be identified in the alternative shufflon of R721 to which pCT's shufflon has most homology. From the sequence analysis all components of the pCT shufflon region are intact and a complete recombinase is present. From the analysis of the plasmid assembly data the pCT shufflon region was found to be present in multiple forms, consistent with site-specific recombination mediated by the pCT shufflon Rci recombinase. This shows that the shufflon region and *pil* locus are actively varying at a level that can be easily detected within a single bacterial culture, indicating that the

shufflon region of the *pil* locus is functional. This differs from all other closely related plasmids (pO113, pO26_vir, pR3521 and pSERB1) as each of these appears to have an inactive shufflon either due to an absent recombinase or an insertion, suggesting that the pCT shufflon is more unique than first thought. Much discussion over the role of this type IV pilus and its shufflon is documented in the literature. Data from Bradley *et al.* (1984) indicated that the thin pilus anchored donor and recipient cells aiding conjugation in liquid media. However the study of pSERB1 by Dudley *et al.* (2006) showed the type IV pilus system increased adhesion of the host bacterium to surfaces and eukaryotic epithelial cells *in vitro*, and allowed more robust biofilm formation. As *E. coli* isolate C159/11 was originally found to persist within the slurry and the floor of the cow sheds during the longitudinal farm study of which it was identified (Teale *et al.*, 2005) these aspects of the type IV pilus may have contributed to the persistence of pCT within the farm environment. The absence of the shufflon recombinase and *pilV* components in pO26_vir, pR3521 and pO113 may suggest that the thin pilus encoded on these plasmids may not have the ability to determine host mating specificity using *pilV* conformational genomic changes, which may have implications for the dissemination of these plasmids both in liquid media, or through mixed species populations when compared to pCT.

There was an absence of any integrons carried on pCT and only a small number of insertion sequences/transposases were identified, unusual for a plasmid of this size. This correlates with the single encoded antibiotic resistance gene, and may suggest the pCT backbone is unfavourable to recombination events or is a potential ancestral backbone rather than a mosaic plasmid, as is believed for plasmid pBP136 (Accession no. AB237782) (Kamachi *et al.*, 2006). From the current sequence data

available, it appears that IncI complex plasmids undergo less recombination and insertions than some of the other plasmid groups such as IncFII (Iredell *et al.*, 2011). This may be associated with the bacterial hosts in which these plasmids reside, transfer between bacterial hosts (Lawrence *et al.*, 1992), or with currently undiscovered plasmid or integron genes or regulators which may influence the frequency of recombination. Similarly, it appeared from the initial annotation that pCT lacked any large post segregational killing genes or addiction operons such as *parA/B* or *kor/mck* found in R64. However, on later re-annotation and further close investigation, a putative toxin-antitoxin CDS, *pndA* (pCT_065), was revealed as a possible toxin but seemed to lack annotation of a regulator (*pndC*) or antisense antitoxin (*pndB*). Alignments of the toxin-antitoxin systems found in plasmid pO113 and R64 revealed that the *pnd* system is encoded on pCT in its entirety. Therefore, is potentially a fully functional post segregational killing system; thus providing a candidate locus which may contribute to the successful persistence of pCT.

Using comparative genomic analysis, regions of pCT with little homology to reference plasmids were also identified. Most notably the putative sigma factor (pCT_066) can only be found in the five other very closely related plasmids and has only limited identity to its homolog SigB in *Yersinia frederiksenii*. Other weak protein matches show some homology to the extra cytoplasmic function (ECF) sigma factors which are small regulatory proteins divergent in sequence to most of the other sigma factors involved in global regulation of transcription. It is also similar to the *Bacteroides thetaiotamicron* VP1-5482 ECF putative RNA polymerase (AE015928). Both examples are chromosomally encoded. Although sigma factors of this group have previously been noted on plasmids, there are few publications on their role or

function. A possible regulatory role, potentially controlling transcription of plasmid or bacterial host chromosomal genes makes this CDS particularly interesting. The rarity of pCT_066 within currently available sequenced plasmids also allowed this element to lend itself to the rapid identification of plasmids closely related to pCT in conjunction with other ORFs. The putative serine-O-acyltransferase gene (pCT_009) also had no homology with any of the other plasmids examined and may be involved in breakdown of serine within host cells.

When comparing the genome of pCT to other *bla*_{CTX-M} encoding plasmids, a lack of any conserved regions outside the antibiotic resistance gene were found. Therefore, it is unlikely that any one genomic factor contributes to the spread of *bla*_{CTX-M} genes on epidemic plasmids or that any one particular genetic factor encoded within these plasmids led to the acquisition of *bla*_{CTX-M} genes from their origin. This correlates with the widely believed hypothesis that multiple acquisitions of *bla*_{CTX-M} genes onto various different plasmids took place. The pCT site at which *ISEcp1-bla*_{CTX-M-14-IS903} has inserted is an unusual pseudogene. This hypothetical protein cannot be found in any of the other reference plasmids or similar plasmids such as pO26_vir, pR3521 or pO113, or from any bacterial chromosomes deposited in Genbank. However, in all the pCT-like plasmids identified, the *bla*_{CTX-M-14} cassette appears to have inserted into this pseudogene as in pCT. The pseudogene has a considerably lower G+C content than the downstream insertion sequence IS903 and to the surrounding IncI plasmid 'backbone' genes *sogL* and *tra* genes (Figure 3.4) perhaps indicating a divergent origin of this pseudogene to the plasmid 'core' genome. Its location upstream of the *ISEcp1* transposase, believed to be responsible for mobilisation of *bla*_{CTX-M-14}

(Nordmann *et al.*, 2008), would suggest that this gene was not mobilised with *bla*_{CTX-M-14}. Therefore, its residence on pCT is likely to pre-date that of *bla*_{CTX-M-14}.

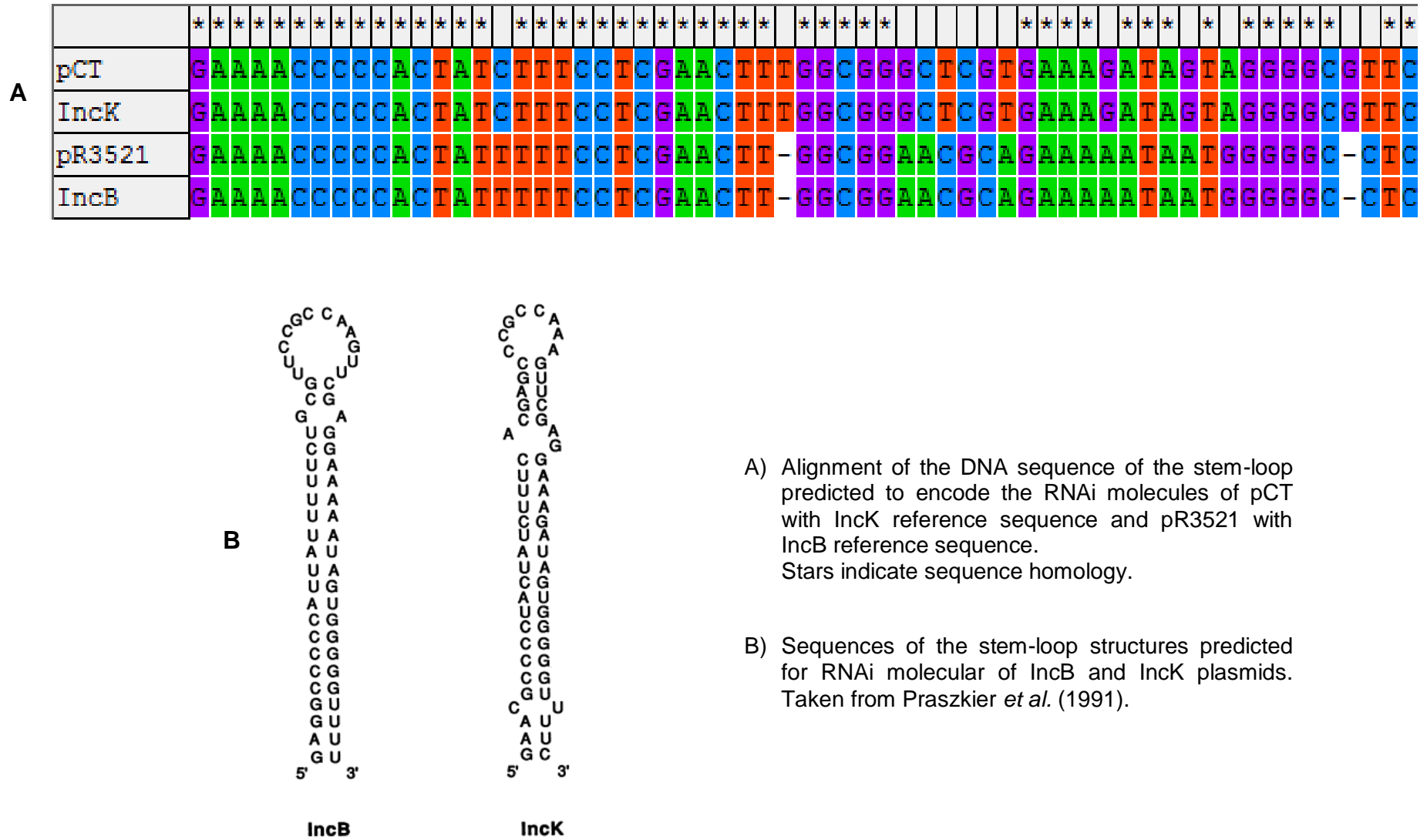
A high degree of homology was found between pCT and four plasmids deposited in Genbank. Relatively little is known about pO26_vir other than its identification in a shiga toxin producing *E. coli* strain 026:HII and the presence of an abundance of virulence genes, including genes for the production of a hydrolase, catalase, and a haemolysin transport protein in addition to the homology found within pCT. More is known about plasmid pO113, which was isolated from another haemolysin producing EHEC 0113:H21 *E. coli* isolate from a patient in Australia (Srimanote *et al.*, 2002; Leyton *et al.*, 2003) and plasmid pR3521 identified in an *E. coli* isolate from a patient in Greece. pR3521 encodes potential virulence genes *ygiJ* and *ygiK* which encode putative adhesive threads, and a large repertoire of resistance genes, found within a cassette situated next to the plasmid transfer operon. Papagiannitsis *et al.* (2011) hypothesised various mechanisms for how this cassette was composed over time, showing that a large resistance cassette is easily constructed on this type of plasmid backbone. While this suggests that plasmid pCT may be able to acquire any number of antibiotic resistance genes such as on pR3521, the question of why pCT has only acquired or stably integrated one antibiotic resistance gene becomes even more prominent. The presence of virulence genes on closely related plasmids also allows the potential for homologous recombination between pCT and these plasmids creating mobile elements carrying both virulence genes and the *bla*_{CTX-M-14} resistance gene.

pCT was found to have most homology with IncB plasmids pO26_vir, pR3251 and TP113 and two of the pCT-like plasmids identified from Australia were also found to

be from the IncB group rather than IncK. Although IncK and IncB plasmids are very closely related (sharing 94% similarity over the genomic replication regions) they are still compatible within the same cell (Praszkier *et al.*, 1991). Both types of plasmid encode an antisense RNA mediated inhibition system used to control replication and copy number; where the RNAi prevents formation of a long distance RNA pseudoknot required for the ribosome binding to *repZ*. In the case of IncK, the RNAi sequesters the *repY* (leader peptide) ribosomal binding site therefore blocking its translation. A lack of RepY prevents activation and translation of *repZ*, resulting in the prevention of pseudoknot formation and subsequent replication of the plasmid. Differences between IncK and IncB are found most importantly in stable stem and loop structures (Figure 3.19) as a similar loop (as found between IncZ and IncI1) would allow crosstalk and interference in copy number control of each plasmid.

When examining the DNA sequences for the loop region of pCT (IncK) and IncB plasmid pR3521, both correspond with the reference sequences provided by Praszkier *et al.*, (1991) (Figure 3.19), suggesting that these closely related plasmids can be very similar at a genetic level (e.g. 83% homologous), yet have differing replication systems due to SNPs in the stem-loop region. This finding also suggests that pCT-like plasmids found in Australian *E. coli* isolates JIE182 and JIE052 are likely to be a pCT backbone with a differing IncB replicon. The most likely explanation for this difference, is a homologous recombination event with another plasmid which has allowed the replacement of one replicon for another (e.g. IncK-IncB or vice versa). Current sequence data may suggest that the pCT backbone has changed from an IncB to an IncK before disseminating in its current form, as most of the known homologous plasmids are of an IncB type and pCT shows considerably

Figure 3.19. Comparison of pCT (IncK) and pR3521 (IncB) antisense loop sequence and structure

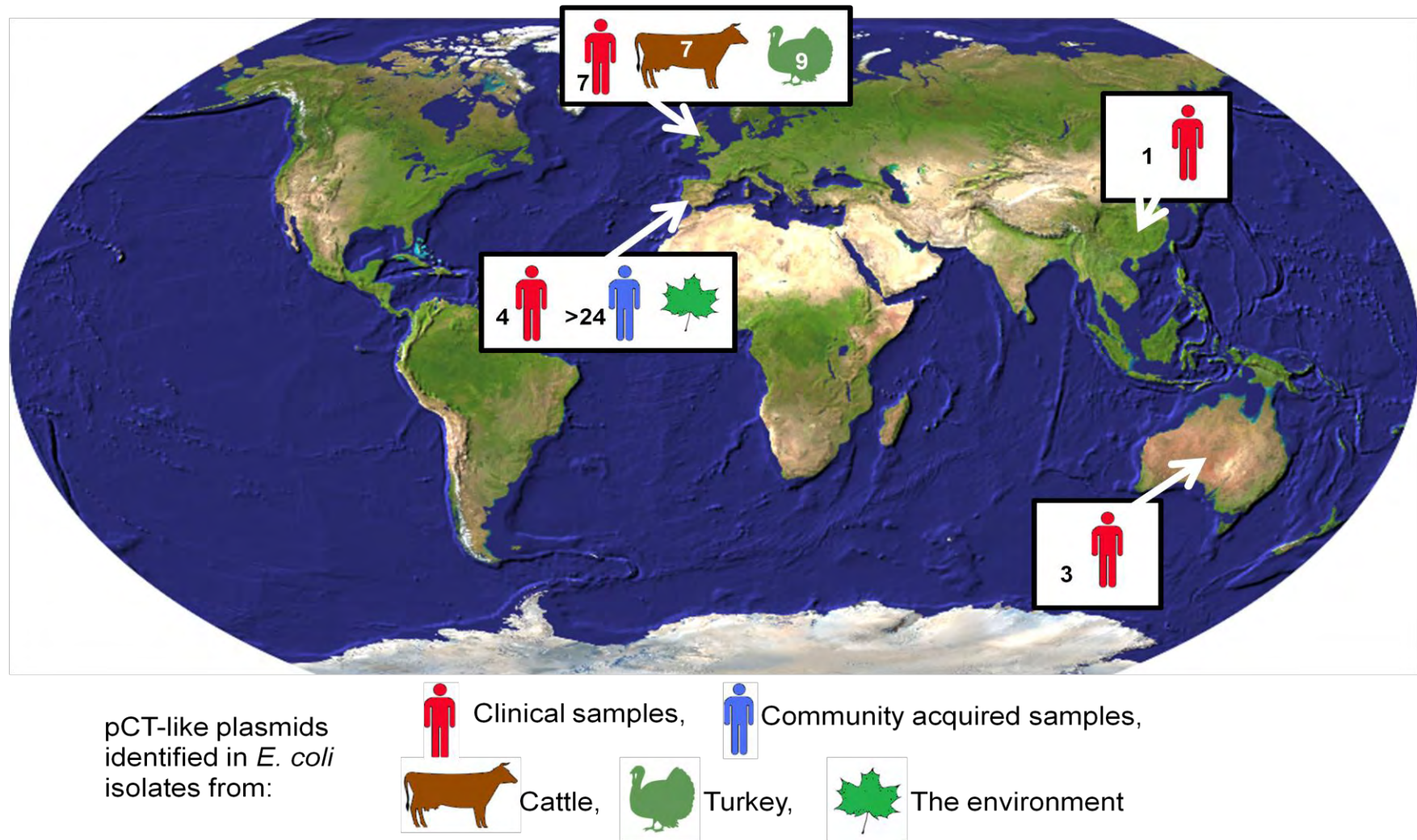


less similarity to IncK plasmid R387. A current lack of other IncK plasmid sequences prevents further investigation of this hypothesis. An incompatibility type switch of this nature would allow co-habitation within a bacterial cell with a plasmid of its original Inc group, or may cause a change in copy number of the plasmid, which in certain environments may be beneficial. Another hypothesis is that pCT-like plasmids predate the evolutionary divergence of IncK and IncB plasmids, or that mutation from one stem loop sequence to another has occurred over time. This explanation seems unlikely as nine SNPs exist throughout the loop sequence. The idea that replicons associated with particular backbones may be interchangeable corresponds with the presence of multiple replicons on a number of sequence plasmids, indicating that it may be beneficial for a plasmid to be able to switch replication and copy number control. It is clear that incompatibility type alone is not sufficient to distinguish between plasmids, to ascertain any evolutionary relationships (Hawkey and Jones, 2009) or to inform about the plasmid backbone. At worst, replicon typing may be misleading, as the exclusion of plasmids with dissimilar Inc types may miss similar backbones within a screen, as demonstrated in this study with IncB pCT-like plasmids.

The complete sequencing of pCT allowed the development of PCR assays designed to amplify genes distributed around the genome. Reactions to amplify *nikB*, *pilN* and the shufflon recombinase (*rci*) were successfully combined creating an efficient and rapid multiplex for high throughput screening of other CTX-M-14 producing isolates for identification of other pCT-like plasmids which share these loci. Initially pCT-like plasmids were identified in bacteria isolated in 2006 from the index farm and from a farm in Lancaster in 2008. More recently this series of six PCR assays have been

used to identify a further five pCT-like plasmids from *E. coli* isolated from cattle, nine from turkeys, and seven clinical *E. coli* isolates from patients in England and Wales (Coldham *et al.*, 2011; Stokes *et al.*, 2011). In my study, 4/5 clinical isolates collected from Spanish hospitals also carried pCT-like plasmids with the identical *bla*_{CTX-M-14} insertion location (Figure 3.20). Since 2000, when CTX-M-14 was first identified in bacteria from Spain, it has rapidly become one of the most commonly detected β -lactamase enzymes in Spanish human and veterinary *E. coli* isolates (Navarro *et al.*, 2007; Blanc *et al.*, 2008). A previous study conducted in Spanish hospitals suggested an association between *bla*_{CTX-M-14} and IncK plasmids. Valverde *et al.*, (2009) isolated an IncK plasmid, later named pRYC105 (from strain FEC383) from at least twenty-five *E. coli* lineages. Isolates were predominantly collected from community acquired infections such as urinary tract infections, and from the environment in different geographical regions across Spain. These authors hypothesised that pRYC105 shared identity with the plasmid isolated in the UK by Liebana *et al.*, (pCT). My study has shown this hypothesis to be correct as pRYC105 was pCT-like by PCR and *nikB* sequencing. Human isolate *E. coli* (8) CH13 isolated in 1998 from China, contained plasmid pOZ174 which encodes the first described *bla*_{CTX-M-14} (Chanawong *et al.*, 2002). pOZ174 was also shown to be pCT-like with an identical *bla*_{CTX-M-14} insertional region, suggesting that pCT has existed in its current form since at least 1998. As China and Spain are locations where CTX-M-14 is the most frequently identified ESBL, further investigation using this molecular test will determine whether pCT is the dominant vector of *bla*_{CTX-M-14} in these areas, and whether pCT has disseminated to other ecosystems or bacterial species.

Figure 3.20. Summary map of the epidemiology of pCT-like plasmid identified from *E. coli* isolates (as of Sept 2011)



From the small sample of veterinary isolates we assembled from the UK (fifteen isolates), it is clear that pCT is not the predominant *bla*_{CTX-M-14} vector as many other Inc type plasmids were found to carry this gene. However all *bla*_{CTX-M-14} carrying IncK plasmids tested to date have been shown to be pCT-like, indicating that pCT is the dominant IncK vector of *bla*_{CTX-M-14}. Both IncB plasmids tested were also shown to be pCT-like. The identical insertion site for *bla*_{CTX-M-14} in each of the investigated pCT-like plasmids strongly suggests a single capture of this β -lactamase gene onto the backbone and subsequent spread of the plasmid. Alternatively, this pseudogene may provide a 'hot spot' for *ISEcp1* insertion, promoting this insertional event, or is mobilisable with the *bla*_{CTX-M-14} cassette.

The alignment and analysis of *nikB* from IncI plasmids was a useful tool to determine the similarity between this plasmid group. It also provided a rapid method for identifying closely related plasmids by a single PCR and DNA sequencing. pCT-like plasmids all demonstrated *nikB* sequence identity of >98% and clustered together with pCT within a phylogenetic tree. Plasmids identified from whole genome comparisons as similar to pCT (pO26_vir, pR3521 and pSERB1) also clustered more closely than the other IncI plasmids. It would appear for this plasmid, a similar *nikB* sequence is a good predictor of whole plasmid homology. Relaxase or *nikB* typing would also provide a suitable locus in recently developed plasmid MLST (pMLST) characterisation, currently not developed for IncK plasmids.

In conclusion, the complete sequence of pCT allowed an understanding of its backbone and facilitated the identification of genes of interest for further study. The sequence revealed the presence of a single antibiotic resistance gene indicating that the success of pCT cannot be attributed to co-selection in the presence of other

antibiotic classes. The sequence analysis also highlighted candidate genes which may contribute to the successful persistence and/or spread of pCT. Subsequent PCR assays successfully showed that pCT-like plasmids are spread globally in bacteria isolated from humans, animals and the environment.

3.9 Further work

At the beginning of this study in October 2008, a major limiting factor was the amount of plasmid sequence data, particularly annotated data available for comparison to pCT. As the study progressed other complete plasmid sequences were deposited in online databases providing an ever growing data source. As more plasmids are sequenced the future integration of new plasmid sequence data into the pCT analysis may elucidate answers to questions raised within my work. For instance, further sequencing of a range of IncK and IncB plasmids would reveal whether pCT is a typical IncK plasmid or whether it relates better to other IncB plasmids as suggested in this work. Whether regions identified for the PCR assays on pCT are truly unique or simply unusual would also be elucidated.

The hypothesis that plasmids can change incompatibility type could be explored further by replacing the pCT *repZ/Y* region with an IncB stem loop DNA region and investigating the changes to plasmid replication. Investigation of whether this change influences copy number could also be more simply examined, initially by comparing the copy number of pCT-like IncB Australian plasmids (pJIE182 and pJIE052) to that of pCT and the other IncK pCT-like plasmids.

The PCR assay designed in this study to identify pCT-like plasmids could be used in a range of environments to establish the true distribution of pCT, as only a small sample was examined here. The PCR assay could be used to interrogate samples

for example from the environment, for the prevalence of pCT-like plasmids which do not carry *bla*_{CTX-M-14}, as currently only CTX-M-14 producing *E. coli* have been examined. The distribution of pCT without *bla*_{CTX-M-14} would give further clues to the evolutionary past of this plasmid, and the potential contribution of the antibiotic resistance gene in the spread of pCT. The PCR assay could also be used to in combination with primers designed to other common antibiotic resistance or virulence genes to reveal whether pCT is able to incorporate large antibiotic resistance cassettes as in pR3521.

Whether the *bla*_{CTX-M-14} insertion element is now stably incorporated on pCT, or whether it is still mobilisable under certain circumstances could be investigated by the addition of another IncK plasmid to a cell already containing pCT. This new plasmid should encode a non-mobilisable antibiotic resistance gene, therefore both the new plasmid and *bla*_{CTX-M-14} could be selected within the growth media and incompatibility should remove pCT. If *bla*_{CTX-M-14} is transferrable this experiment would select for its translocation onto the new IncK plasmid. An experiment of this type would also inform on whether the pseudogene (the gene in which *bla*_{CTX-M-14} is inserted) is able to mobilise with this resistance gene, whether it provides a 'hot spot' for insertion, or whether its association with the *bla*_{CTX-M-14} on the pCT-like plasmids is incidental.

Future work could also attribute function to the hypothetical proteins identified in the pCT annotation by systematic inactivation and biological investigation of each open reading frame, as any of these CDSs could potentially have fundamental roles in plasmid biology.

3.10 Key findings

- The plasmid pCT genome was completely sequenced, annotated and interrogated for candidate genes which may contribute to the spread and persistence of this plasmid.
- The *bla*_{CTX-M-14} gene was found to be the only resistance gene encoded on the plasmid and β -lactam resistance, the only transferrable phenotype.
- Comparisons of pCT to other *bla*_{CTX-M} carrying plasmids revealed no commonality between these plasmids outside of the β -lactamase genes.
- Comparisons of pCT with closely related plasmids revealed conserved genomic regions and those pCT regions unusual or unique when compared to current literature. These included the presence of a putative sigma factor and a functional shufflon within the *pil* locus.
- A PCR assay to detect pCT-like plasmids was developed and validated using UK cattle *E. coli* isolates, identifying two pCT-like plasmids.
- Eight pCT-like plasmids were identified in *E. coli* clinical isolates from Europe, Australia and Asia including one isolate from 1998 and a widely distributed previously characterised Spanish plasmid pRYC105. These data suggest global distribution of pCT in bacteria from humans, animals and the environment.
- IncK and IncB pCT-like plasmids were identified.
- The pCT-like plasmids were all found, like pCT, to be transferrable through conjugation and to confer resistance to β -lactams only. Other similar plasmid sequences extracted from Genbank indicate that similar plasmids are able to acquire multiple antibiotic resistance genes and virulence genes.
- *nikB* sequencing was successfully used to compare plasmids and was found to be a useful typing tool in this study.

Chapter 4:

Factors affecting pCT persistence, and fitness of its bacterial hosts

4. Factors affecting plasmid pCT persistence, and fitness of its bacterial hosts

4.1 Background

Assessment of the fitness cost or benefit of plasmids upon their bacterial host has been notoriously complex (Petersen *et al.*, 2009), due in part to the ever changing relationship between plasmids, the bacterial host and environmental conditions. The cost/benefit of genes in the plasmid accessory genome such as antibiotic resistance genes must also be considered (Lenski *et al.*, 1994). Analysis of the pCT genome showed the presence of a single antibiotic resistance gene (*bla*_{CTX-M-14}), but no other genes associated with plasmid conferred fitness costs or benefits were identified.

4.2 Hypotheses and Aims

Plasmid pCT does not confer a fitness burden on host bacterial cells and increases the evolutionary fitness of the host by modifying its phenotype, therefore ensuring the persistence of both plasmid and host. In addition, aspects of the pCT biology such as stability and transfer rate contribute to the plasmid's success.

The aims of this section were to:

- Measure the phenotypic effects conferred when pCT is added to four different bacterial host strains,
- Investigate whether pCT confers a growth fitness cost or benefit when host strains (+/- pCT) were competed in a 1:1 ratio *in vitro*,
- Investigate basic plasmid biological functions of pCT, such as transfer and persistence which may affect plasmid success,
- Investigate whether sub-inhibitory concentrations of cefotaxime affect pCT transfer rates.

4.3 The effect of the addition of pCT on bacterial host phenotype

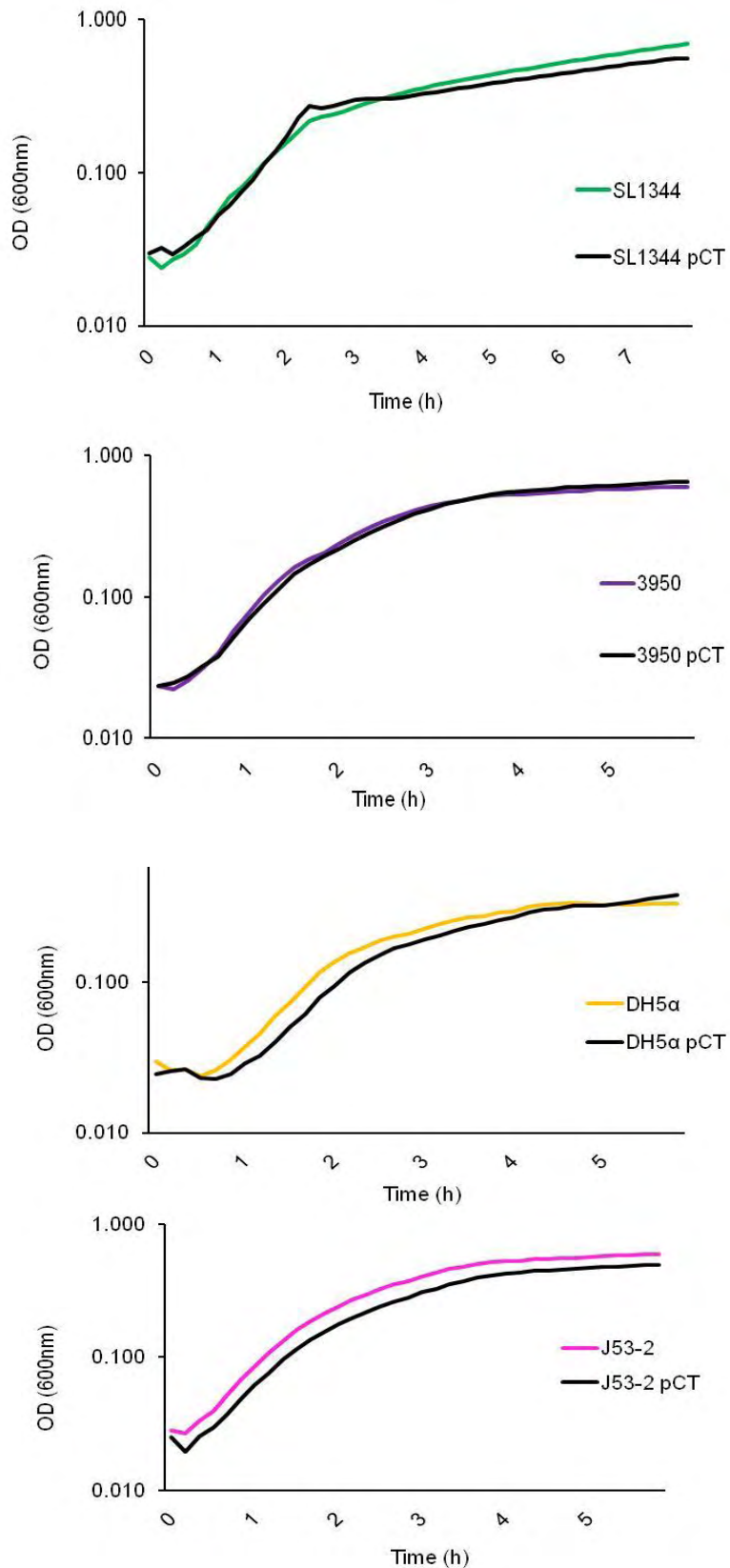
4.3.1 Growth of bacterial host strains +/- pCT

The growth rates of all four parental strains *E. coli* DH5 α (I825), J53-2 (I847) and 3950 (I823) and *Salmonella* Typhimurium SL1344 rif^R (L1078) were compared with and without pCT (I755, I855, I834 and L1079). To minimise co-evolution of host and plasmid or changes to the parental strain, each strain was removed from the -80°C freezer, cultured on agar, then passaged once only without the addition of antibiotic. These cultures were used to inoculate LB broth (three biological and four technical repeats) and the optical density (600 nm) was measured using the automated FLUOstar OPTIMA (BMG Labtech, UK) for a period of twelve hours. The addition of pCT had no statistically significant effect on the growth rate or generation times of the host strains during logarithmic phase (Figure 4.1, Appendix 4). However *E. coli* DH5 α pCT and J53-2 pCT had consistently extended lag phases (an additional ~20 minutes and ~12 minutes respectively) when compared to parental strain *E. coli* DH5 α .

4.3.2 Co-culture of bacterial host strains +/- pCT

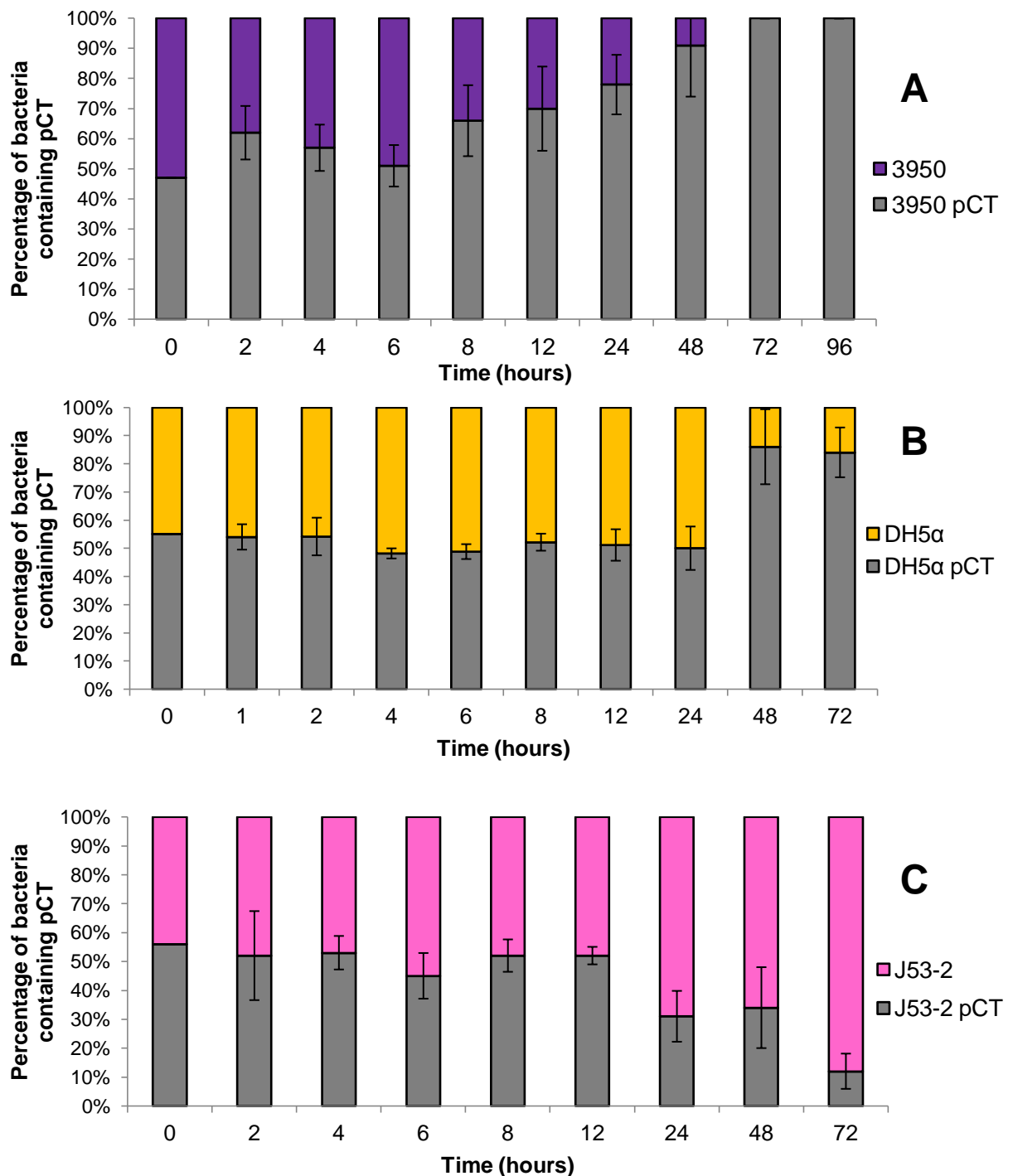
Host strains *E. coli* 3950, *E. coli* DH5 α and *E. coli* J53-2 with and without pCT were mixed in a 1:1 ratio *in vitro* and cultured for 72 hours with four passages, in the absence of β -lactam pressure on three separate occasions. The proportion of bacteria carrying pCT at each time point was calculated allowing the measurement of the progression or 'success' of pCT in each population. In host strain *E. coli* 3950 the proportion of bacteria carrying pCT steadily increased over time from 50% at time zero to 100% after 72 hours (Figure 4.2A). In *E. coli* DH5 α , pCT remained in ~50% of the bacteria until the second passage where in all three biological replicates the proportion of bacteria carrying pCT increased to ~84% (Figure 4.2B).

Figure 4.1. Growth kinetics of host strains +/- pCT



The growth kinetics of each bacterial host strain +/- pCT were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Appendix 4).

Figure 4.2. The proportion of pCT carrying strains within a population



The percentage of plasmid pCT carrying bacteria was calculated over time when *E. coli* strains +/- pCT were co-cultured in a 1:1 ratio *in vitro* (Appendix 5). A, The proportion of pCT carrying bacteria in a 3950 +/- pCT co-culture; B, The proportion of pCT carrying bacteria in a DH5α +/- pCT co-culture; C, The proportion of pCT carrying bacteria in a J53-2 +/- pCT co-culture. Cultures were passaged into fresh broth at hour 12, 24, 48 and 72.

In host *E. coli* J53-2 a different pattern was observed, the proportion of bacteria carrying pCT reduced after the first passage at 12 hours (Figure 4.2C) to 30% at 24 hours and 12% at 72 hours indicating loss of the plasmid or a considerable pCT fitness burden on *E. coli* J53-2 cells (Appendix 5).

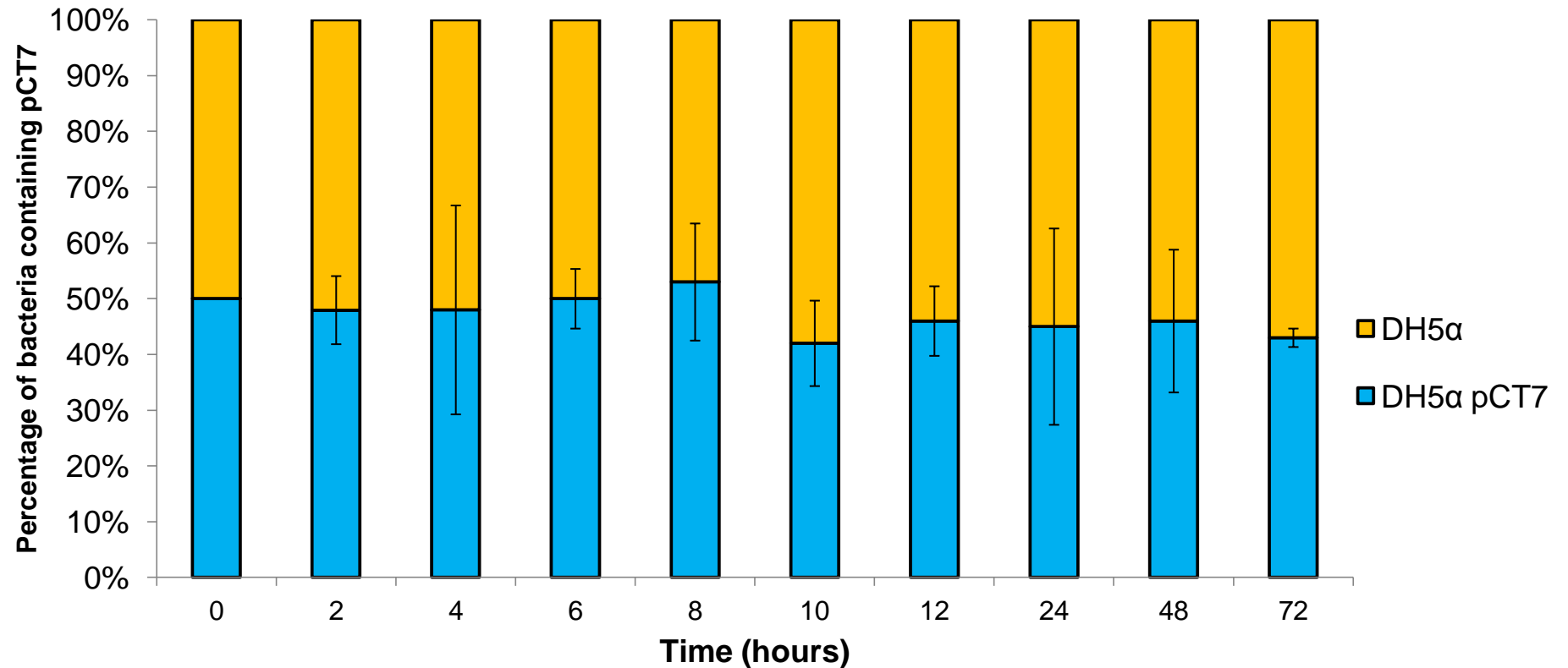
4.3.3 In vitro competition between *E. coli* DH5α and DH5α pCT (non-transferrable)

In order to directly compare the fitness of pCT carrying bacterium compared with a plasmid free counterpart, a non conjugative pCT (pCT7; described and characterised in Chapter 6) was used to remove the confounding factor of plasmid transfer within the competition assay. *E. coli* DH5α pCT7 and DH5α without plasmid were competed in a 1:1 ratio *in vitro* over a 72 hour period. At each time point the strains remained in a 1:1 ratio (Competition index of DH5α pCT7 = 0.99829) suggesting that the presence of pCT within *E. coli* DH5α confers neither a fitness cost nor a fitness benefit in the absence of antibiotic selective pressure (Figure 4.3). As pCT7 could not be transferred by conjugation or transformation to the other host backgrounds the pCT7 fitness burden on *E. coli* 3950 or *E. coli* J53-2 of pCT7 could not be assessed.

4.3.4 The ability of bacterial host strains (+/- pCT) to cause infection

The effect of pCT upon the ability of bacterial host strains to cause infection was assessed using two models of infection. The first measured the ability of *S. Typhimurium* SL1344 rif^R and *S. Typhimurium* SL1344 rif^R containing pCT (L1079) to adhere to, and invade, human embryonic intestinal cells (INT-407) within a monolayer. The second model measured the rate at which nematode *C. elegans* were killed when fed on cultures of *S. Typhimurium* SL1344 rif^R or *E. coli* 3950 +/- pCT.

Figure 4.3. Pairwise competitive growth *in vitro* between *E. coli* DH5 α and DH5 α containing pCT7 (non-conjugative pCT)



Competition index of DH5 α pCT7 at 12 hours (before first passage) = 0.94448, $P = 0.742$ (no significant change)

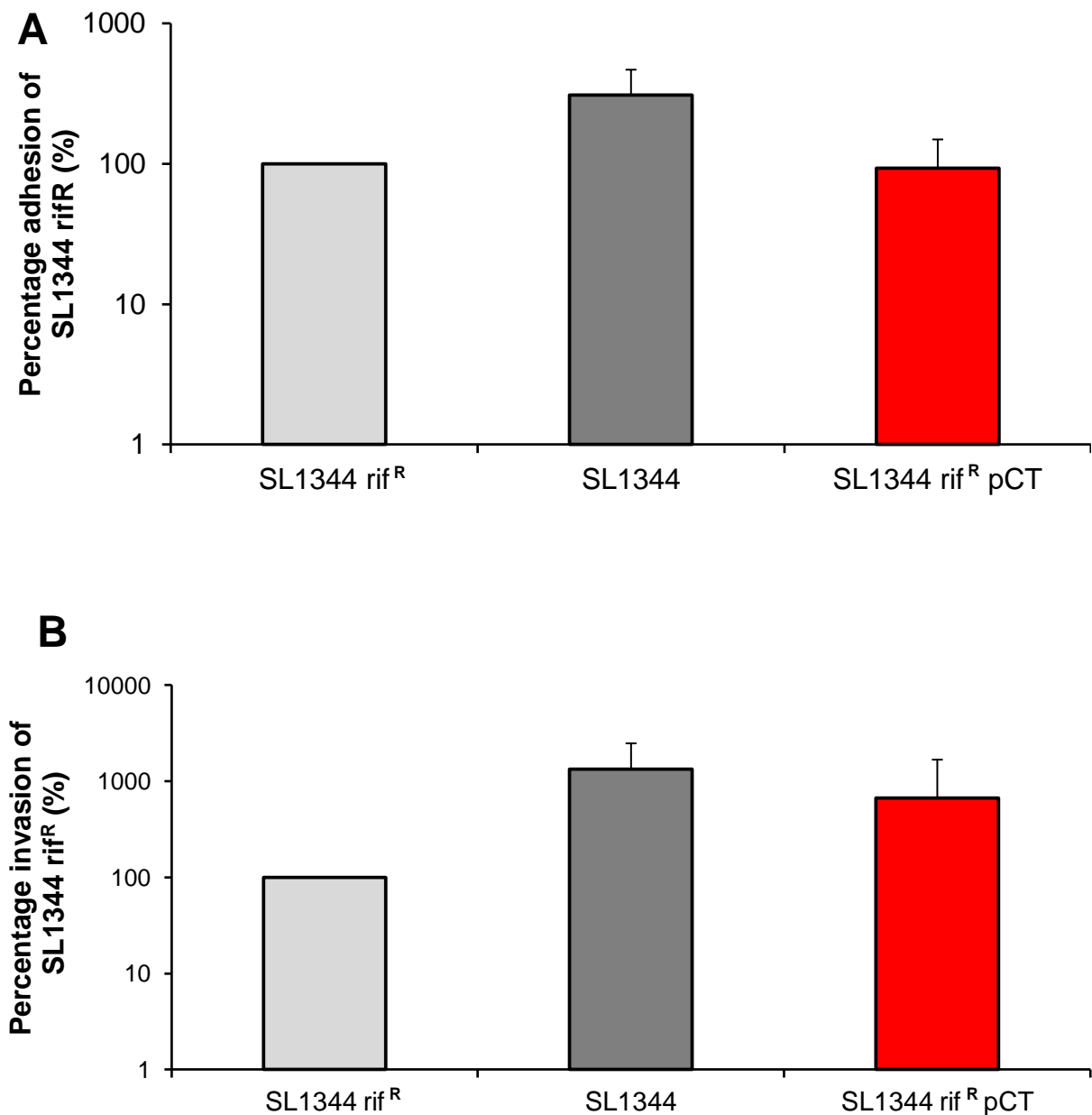
Competition index of DH5 α pCT7 at 72 hours (after three passages) = 0.99829, $P = 0.359$ (no significant change)

The percentage of bacteria carrying plasmid pCT7 over time was investigated when *E. coli* DH5 α +/- pCT7 were co-cultured in a 1:1 ratio *in vitro*. Cultures were passaged at hour 12, 24 and 48. The competition index was calculated from averages generated from at least three separate experiments.

S. Typhimurium SL1344 was chosen as the primary host strain to investigate pathogenicity as its use in both models has been well documented in the literature and has been optimised in our laboratory. *E. coli* 3950 was selected as a representative pathogenic *E. coli* strain (the natural host of pCT) to control for any *S. Typhimurium* specific effects within these assays. The ability of *S. Typhimurium* SL1344 *rif^R* (*rpoB* mutation) to cause infection was also compared to that of wild-type SL1344. The latter strain was used as a control in all experiments due to the large amount of previous data available for this strain in our laboratory (Blair *et al.*, 2009; Bailey *et al.*, 2010). In each experiment if positive control strain *S. Typhimurium* SL1344 was not found to associate with INT-407 cells within predetermined confidence intervals (1.2×10^6 to 1.1×10^7 CFU/ml), the experiment was rejected and repeated. Rifampicin resistant *S. Typhimurium* SL1344 was slightly attenuated when compared to wild-type SL1344 in its ability to adhere to and invade tissue culture cells although not to a statistically significant level (Adhesion, $P = 0.10$, Invasion, $P = 0.11$) (Figure 4.4, Appendix 6). However, *S. Typhimurium* SL1344 *rif^R* killed *C. elegans* at a significantly faster rate than SL1344 ($TD_{50} = 3.5$ vs 3.9 $P = 0.015$) (Figure 4.5, Appendix 7). Therefore, when assessing the effects of pCT on the ability of host *S. Typhimurium* SL1344 *rif^R* to cause infection, comparisons were made to the direct parental strain (SL1344 *rif^R*; L1078) and not wild-type SL1344 (L354).

The number of *S. Typhimurium* SL1344 *rif^R* and *S. Typhimurium* SL1344 *rif^R* pCT bacteria able to adhere to and invade the INT-407 cells was not significantly different (Adhesion, $P = 0.93$; Invasion, $P = 0.23$). Addition of pCT had no effect on the rate at which host strain *E. coli* 3950 killed the nematodes in the *C. elegans* model ($P = 0.89$) and the TD_{50} of the nematodes remained unchanged ($TD_{50} = 4.76$ days)

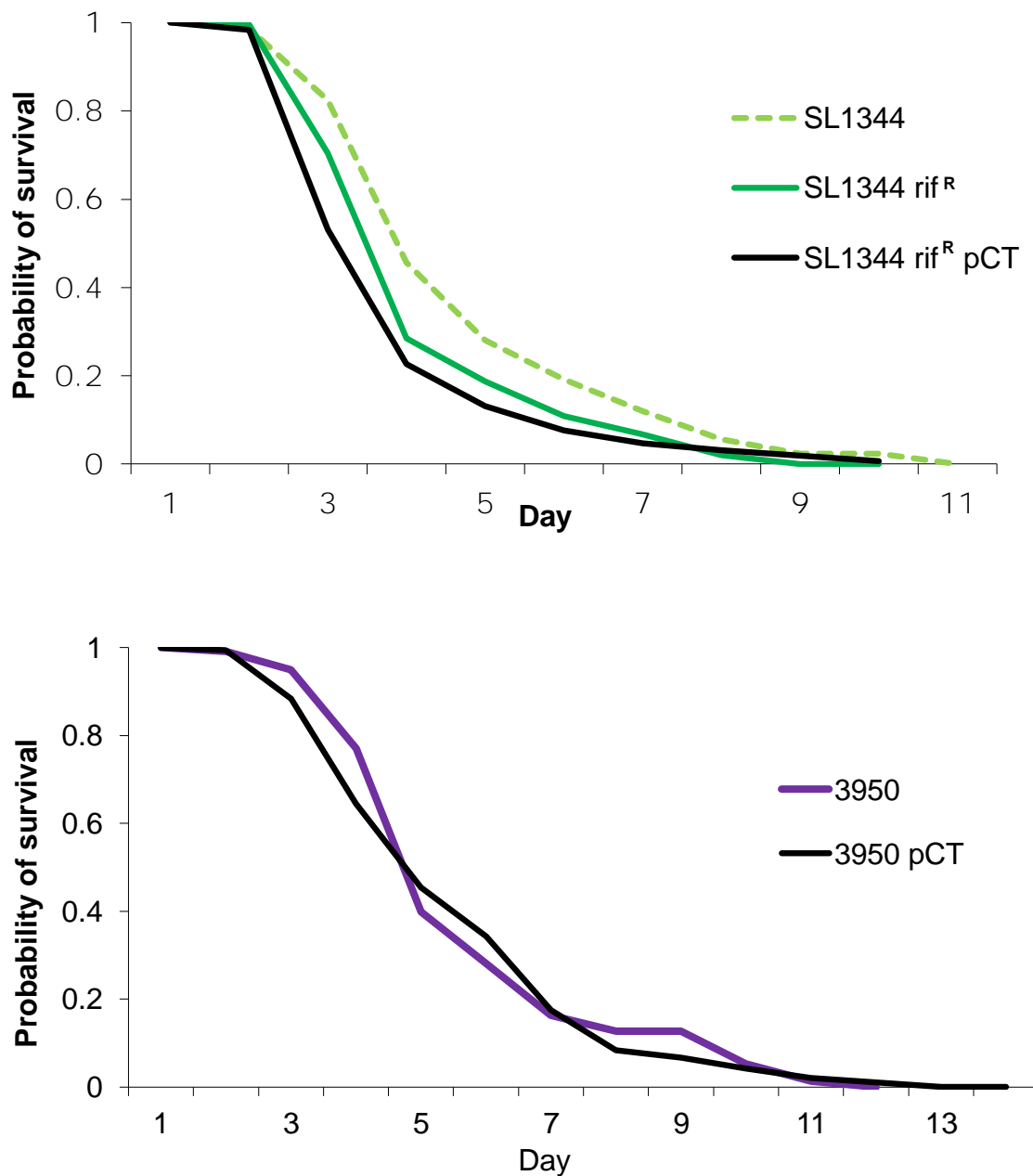
Figure 4.4. The ability of *S. Typhimurium* SL1344, SL1344 rif^R and SL1344 rif^R containing pCT to adhere to and invade human intestinal cells



The number of *S. Typhimurium* SL1344 and SL1344 rif^R pCT bacterium to adhere to (A) and invade (B) INT-407 human intestinal cell is represented as a percentage of the number of *S. Typhimurium* SL1344 rif^R able to do so under test conditions (Appendix 6). Data are displayed as a mean of three separate experiments all performed with four technical repeats.

Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant are represented with an asterix.

Figure 4.5. The ability of bacterial hosts +/- pCT to cause infection in *C. elegans*



Survival curves were constructed using a Kaplan Mier calculation showing the probability of *C. elegans* survival each day when fed continuously on the tested bacterial strains.

A significance difference was found in the killing rate between *S. Typhimurium* SL1344 rif^R and *S. Typhimurium* SL1344 ($P = 0.0154$). A significant difference in killing rate was defined as a P value <0.05 (Appendix 7).

(Figure 4.5, Appenix 6). The addition of pCT to *S. Typhimurium* SL1344 rif^R also had no significant effect on the hosts ability to cause infection in the *C. elegans* model, although the average TD₅₀ was reduced from 3.5 to 3.15 days.

4.4 Plasmid pCT persistence and transfer

4.4.1 Persistence of pCT in bacterial host cells

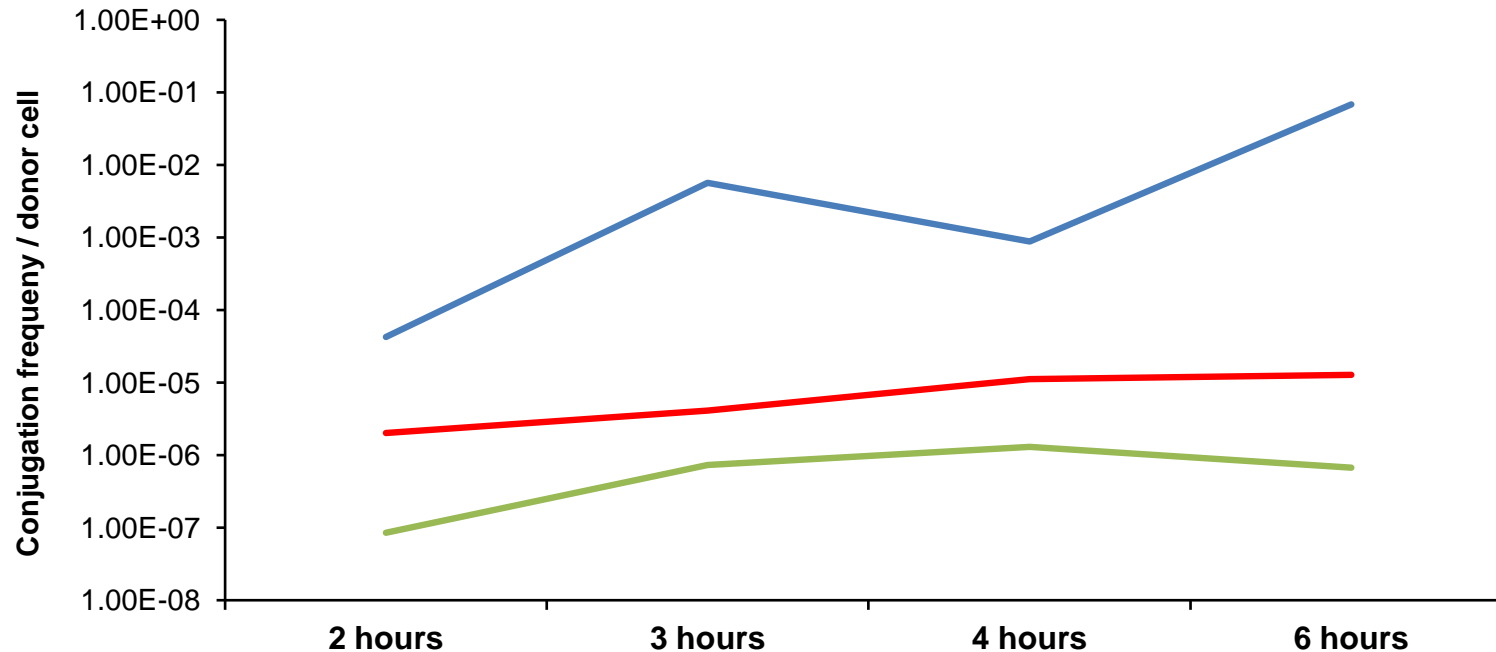
The stability of plasmid pCT was measured by growing pure cultures of each pCT containing strain (*E. coli* C159/11; *E. coli* DH5 α pCT; *E. coli* J53-2 pCT and *S. Typhimurium* SL1344 rif^R pCT) in LB broth. Cultures were grown over a 96-hour period including four passages, calculated from viable counts to be ~70 generations, with and without antibiotic selection (cefotaxime 8 μ g/ml). The proportion of bacteria containing pCT was calculated every two hours during the first 12 hours and after each passage on three separate occasions. For each strain, pCT was stably maintained in 100% of the bacteria examined, both with and without cefotaxime and at each time point measured.

4.4.2 pCT transfer rates

4.4.2.1 *Optimisation of pCT conjugation methods*

In order to establish the optimum time required to measure differences in the rate of transfer of pCT, initial experiments determined conjugation frequencies at 2 hours, 3 hours, 4 hours and 6 hours into various host strains (Figure 4.6; Appendix 8). At 2 hours the frequency of pCT transfer to recipient strain *S. Typhimurium* SL1344 rif^R was too low to provide reliable data, as the number of colonies obtained on agar plates when culturing from the conjugation mixture was less than 30 per plate. The number of transconjugants isolated from the mating pair with the highest rate of transfer, donor *E. coli* C159/11 to recipient *E. coli* DH5 α , after 4 and 6 hours were

Figure 4.6. pCT conjugation frequency time optimisation on solid media



The pCT conjugation frequencies were measured from donor strains *E. coli* C159/11 and *E. coli* DH5α during a 2,3,4 and 6 hour period to optimise the the timing for future conjugation experiments (Appendix 8) on a minimum of three separate occasions.

Blue line = Conjugation frequencies of pCT from donor *E. coli* C159/11 to an *E. coli* DH5α recipient

Red line = Conjugation frequencies of pCT from donor *E. coli* C159/11 to a *S. Typhimurium* SL1344 rif^R recipient

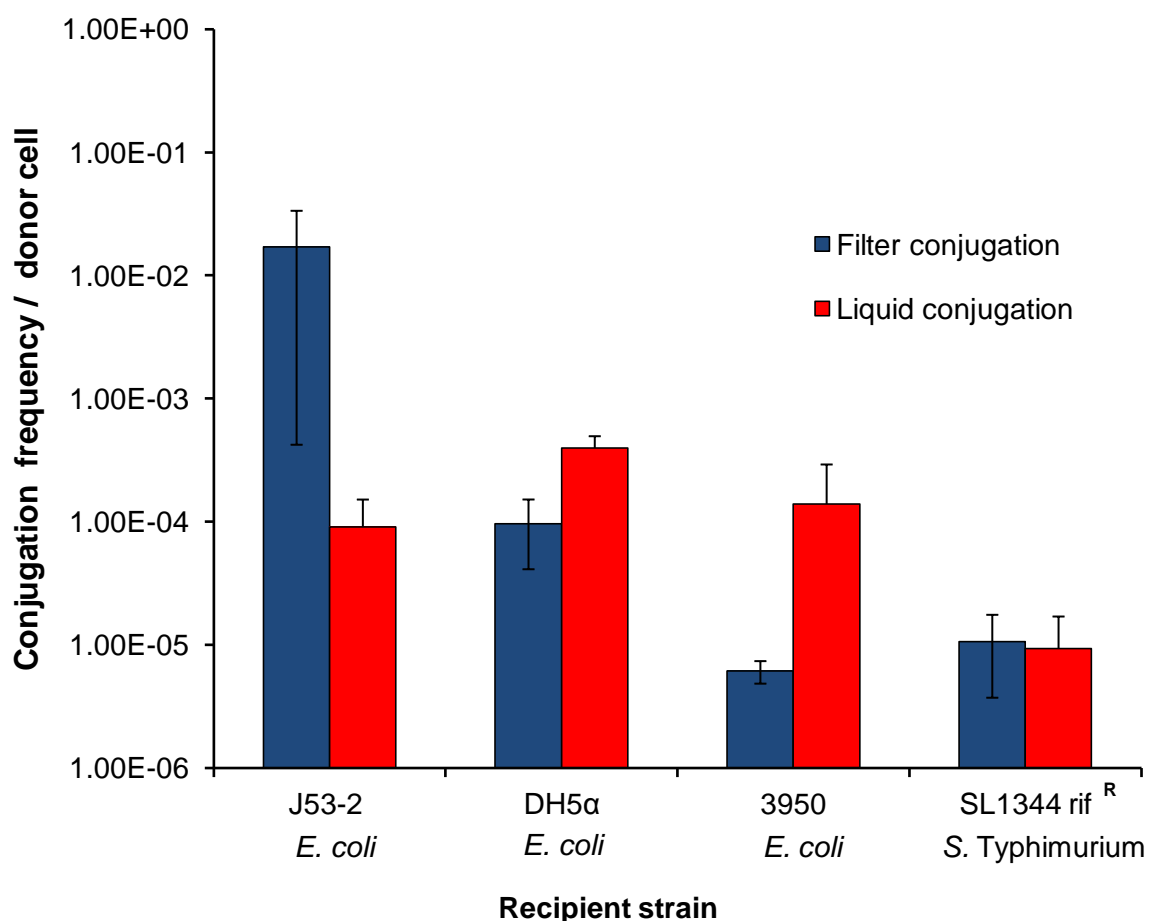
Green line = Conjugation frequencies of pCT from donor *E. coli* DH5α pCT to a *S. Typhimurium* SL1344 rif^R recipient

near to the number of recipient cells suggesting pCT saturation within the population. It is also likely by this time transconjugants may themselves donate pCT to pCT-free bacterial cells. Therefore, 3 hours was chosen for all further conjugation frequency experiments.

4.4.2.2 *Frequency of pCT conjugative transfer to various recipient bacterial strains*

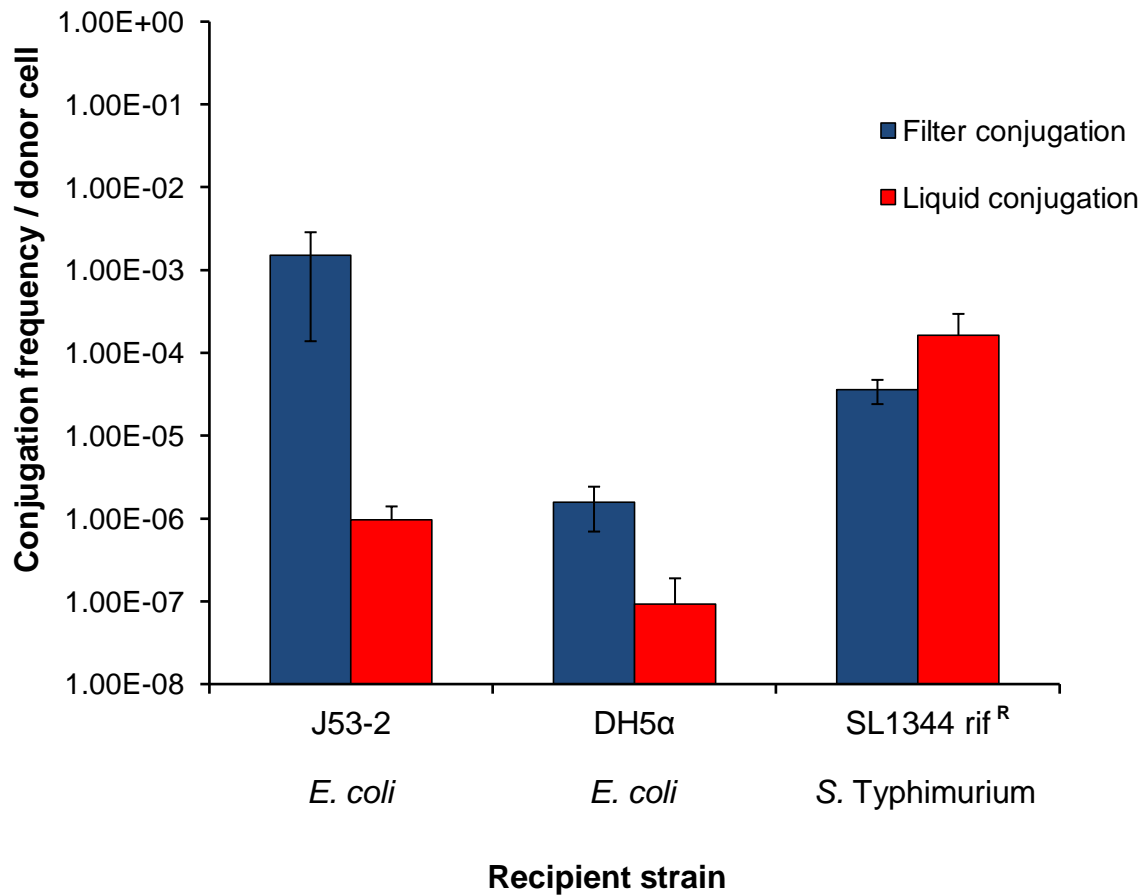
The pCT transfer frequency was measured from donor strain *E. coli* DH5 α to recipient strains *E. coli* J53-2 (I847), *E. coli* DH5 α rif^R (I754) and *E. coli* 3950 (I823) and *S. Typhimurium* SL1344 rif^R (L1078) on filters placed on LB agar plates (filter) and in LB broth (liquid). Each experiment was repeated on at least three separate occasions. The conjugation frequency of pCT ranged from 7.44×10^{-6} to 1.49×10^{-2} per donor cell over the three hour period. pCT transferred at the highest rate to recipient *E. coli* J53-2 on a filter, in liquid the conjugation frequency was reduced two fold. A large difference was also found between the conjugation frequency of pCT on solid and liquid media to recipient *E. coli* 3950 where the conjugation was at a higher rate in liquid than on a filter. pCT transfer to *S. Typhimurium* recipient SL1344 rif^R was at similar levels in liquid and on a filter, as were the conjugation frequencies into recipient *E. coli* DH5 α rif^R (Figure 4.7, Appendix 9). The conjugation frequency of pCT from donor strain *E. coli* C159/11 to recipients *E. coli* DH5 α rif^R and *E. coli* J53-2 were consistently lower than from donor *E. coli* DH5 α (Figure 4.8, Appendix 9), however, pCT transfer from *E. coli* C159/11 to recipient *S. Typhimurium* SL1344 rif^R was at a higher frequency than from donor *E. coli* DH5 α both on a filter and in broth. These data suggest both donor and recipient strain specific effects, but that pCT can be transferred between a range of bacterial strains including a *S. Typhimurium* strain.

Figure 4.7. Conjugation frequencies of pCT from donor *E. coli* DH5α to various recipients on solid and in liquid media



The conjugation frequency of pCT from donor bacterial strain *E. coli* DH5α to recipient strains *E. coli* J53-2, *E. coli* DH5α, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R was measured over a three hour time period, on a filter (blue bars) and in liquid media (red bars). Conjugation frequencies were calculated from the mean of at least three separate experiments (Appendix 9A).

Figure 4.8. Conjugation frequencies of pCT from donor *E. coli* C159/11 to various recipients on solid and in liquid media



The conjugation frequency of pCT from donor bacterial strain *E. coli* C159/11 to recipient strains *E. coli* J53-2, *E. coli* DH5α and *S. Typhimurium* SL1344 rif^R was measured over a three hour time period, on a filter (blue bars) and in liquid media (red bars). Conjugation frequencies were calculated from the mean of at least three separate experiments (Appendix 9B).

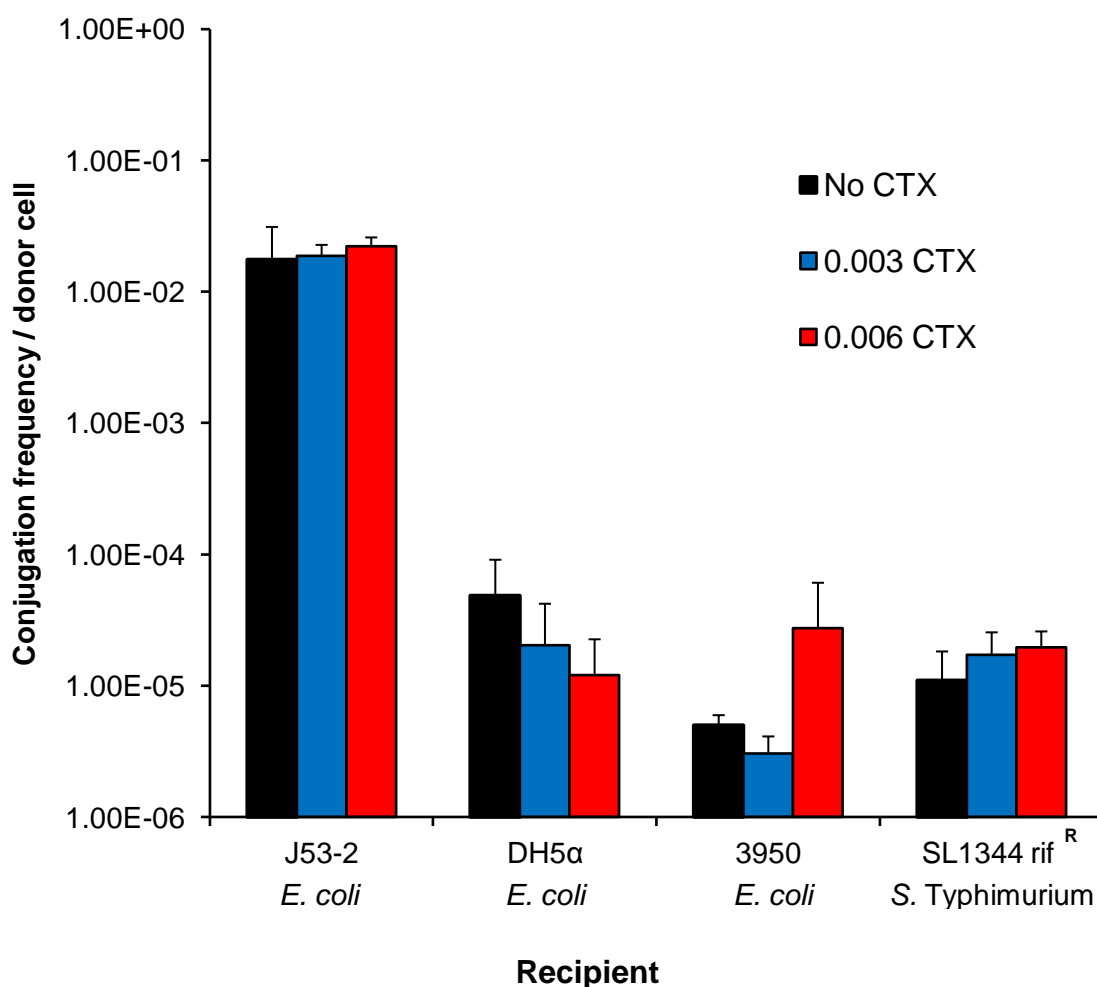
4.5 pCT conjugation rates in the presence and absence of cefotaxime

The frequency of pCT transfer from donor *E. coli* DH5 α to recipients *E. coli* J53-2, *E. coli* 3950, *E. coli* DH5 α rif^R and *S. Typhimurium* SL1344 rif^R were measured in the presence of sub-inhibitory concentrations of cefotaxime (0.003 μ g/ml and 0.006 μ g/ml) and compared to the same donor and recipient pairs in the absence of antibiotic. The pCT conjugation frequencies to all recipient strains were not significantly different in the presence of sub-inhibitory concentrations of cefotaxime either on a filter (Figure 4.9, Appendix 10) or in liquid (Figure 4.10, Appendix 11), suggesting that the presence of sub-inhibitory concentrations of antibiotics does not impact conjugation rates of pCT.

4.6 Discussion

Traditionally, the relative fitness of bacterial strains has been investigated by comparing their growth rates and generation times in pure culture, as differential growth has been shown to be a strong indicator of bacterial fitness (Andersson and Hughes, 2010). Therefore, in my study the fitness cost/benefit of pCT upon three 'pCT naive' bacterial hosts *E. coli* DH5 α , *E. coli* J53-2 and *S. Typhimurium* SL1344 rif^R, and on *E. coli* 3950 (derived from C159/11 and pCT 'cured') was initially assessed by comparing growth kinetics. The addition of pCT had no significant effect on the generation time of the four host strains, revealing that in the absence of antibiotic pressure, pCT conferred neither a growth benefit or growth defect on the host bacterium. These findings contradict those of Zünd and Lebek (1980), who showed that the majority of large plasmids (>80 kb) increased the generation times of their new host strains.

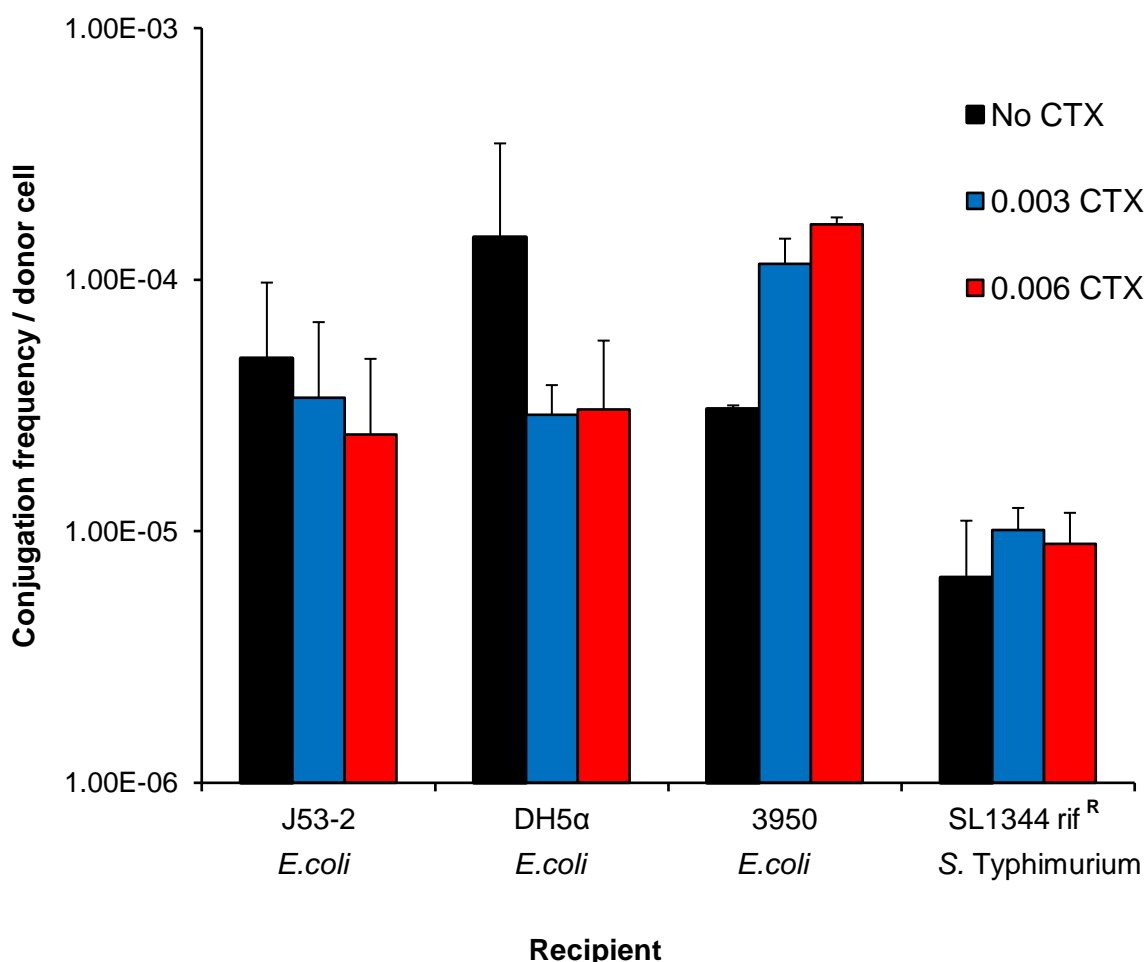
Figure 4.9. Conjugation frequencies on solid media of pCT in the presence of sub-inhibitory concentrations of cefotaxime



The conjugation frequency of pCT in the presence of sub-inhibitory concentration of cefotaxime (CTX) was compared to conjugation frequency in the absence of antibiotic. Frequencies were measured from donor bacterial strain *E. coli* DH5α to recipient strains *E. coli* J53-2, *E. coli* DH5α, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R on a filter placed on an agar containing either no cefotaxime (Black bars); 0.003μg/ml cefotaxime (Blue bars) or 0.006μg/ml cefotaxime (Red bars).

Conjugation frequencies were calculated from the mean of at least three separate experiments (Appendix 10). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant are represented with an asterix.

Figure 4.10. Conjugation frequencies in liquid media of pCT in the presence of sub-inhibitory concentration of cefotaxime



The conjugation frequency of pCT in the presence of sub-inhibitory concentration of cefotaxime (CTX) was compared to conjugation frequency in the absence of antibiotic. Frequencies were measured from donor bacterial strain *E. coli* DH5α to recipient strains *E. coli* J53-2, *E. coli* DH5α, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R in LB broth containing either no cefotaxime (Black bars); 0.003μg/ml cefotaxime (Blue bars) or 0.006μg/ml cefotaxime (Red bars).

Conjugation frequencies were calculated from the mean of at least three separate experiments (Appendix 11). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant are represented with an asterix.

My data is also inconsistent with the consensus within the literature that before a period of co-evolution, an initial fitness and growth burden will be conferred by a plasmid to a naive host (Bouma and Lenski, 1988; Modi and Adams, 1991; McDermott *et al.*, 1993). An increased *E. coli* lag time due to carriage of plasmids (as shown in *E. coli* DH5 α pCT and J53-2 pCT) has been observed previously by others, for example the addition of pBluescript (2691 bp) was shown to increase the lag phase of *E. coli* XL1 blue cells from 90 to 126 minutes (Smith and Bidochka, 1998). The *E. coli* DH5 α strain used in my study has had a considerable number of passages and laboratory modification (Table 2.1), therefore, it may be that these strains were the most susceptible to changes in growth, and therefore a good indicator of fitness effects, but perhaps unrepresentative of natural host strains.

Another commonly used method to investigate comparative fitness of bacterial strains is pair-wise competitive growth, where two strains compete for resources in the same culture (Pope *et al.*, 2010). Direct competition of *E. coli* DH5 α pCT against its parental strain *E. coli* DH5 α was not possible due to transfer of pCT to plasmid free cells. Therefore, a non-transferable pCT mutant (pCT7, *traXY::aph*; Chapter 6) was used. These experiments showed that *E. coli* DH5 α cells with and without pCT(7) remained in a 1:1 ratio for 72 hours when directly competed *in vitro*. This suggests that non-transferable pCT conferred neither a fitness cost nor benefit to *E. coli* DH5 α cells. Although the costs associated with conjugation could not be taken into account using pCT7, the experimental method allowed for simple assessment of the plasmid burden/benefit on the host bacterial cell, and was considered simpler than other protocols which adjust for the ratio of transconjugants within the culture (Yates *et al.*, 2006; Subbiah *et al.*, 2011). To investigate how plasmid transfer

affected the proportion of pCT harbouring cells in a population over time, plasmid free and plasmid bearing bacteria strains were placed in a 1:1 ratio in the absence of antibiotic pressure, this time using wild-type pCT. By 72 hours pCT was present in the majority of both *E. coli* DH5 α and *E. coli* 3950 cells. As *E. coli* DH5 α pCT(7) did not outcompete *E. coli* DH5 α , it is likely that the reduction in the number of plasmid free bacteria is due to transfer of pCT and subsequent spread through the population rather than expansion of DH5 α pCT. The reduction in *E. coli* J53-2 plasmid carrying cells when co-cultured with *E. coli* J53-2 indicated a fitness burden conferred by pCT to the host rather than plasmid 'loss', as when J53-2 pCT was cultured alone, pCT was stably maintained in all bacteria over the same time period.

Why pCT has a detrimental effect on *E. coli* J53-2 fitness and not *E. coli* 3950 or *E. coli* DH5 α is unclear. Several other studies have highlighted strain or species-specific differences in fitness costs of particular plasmids, however, no explanations or hypotheses have been provided (Enne and Bennett, 2006; De Gelder *et al.*, 2007; Subbiah *et al.*, 2011). The simplest explanation is a differential pCT copy number in different bacterial host strains, as the reduction of copy number has been shown to reduce the metabolic cost conferred to the host cell (Bentley *et al.*, 1990; Smith and Bidochka, 1998). The increase in the number of *E. coli* DH5 α containing pCT cells between 24 hours (51%) and 48 hours (84%) when *E. coli* DH5 α and *E. coli* DH5 α pCT were co-cultured, also appeared to be strain-specific. This may be suggestive of a pCT or host encoded plasmid transfer control mechanism. While potential *Enterococcus spp.* recipients can produce pheromones which induce conjugation of certain plasmids from the donor strain (Wardal *et al.*, 2010), the initiation of conjugative transfer and the role of cell density, quorum-sensing mechanisms and

constitutively expressed genes in other species or IncI plasmids has not been investigated. The pCT genome contains pCT_010 (annotated originally as *yafB*). This CDS closely resembles genes that encode conjugation repressors *ydbA* in IncI plasmid R64 and *finO* of IncF plasmids (Frost *et al.*, 1994; Sampei *et al.*, 2010). Therefore, there may be differential regulation or expression of this pCT gene in different host strains, which would modify the nature of pCT dissemination (and conjugation frequency) in a naive population (Meynell and Datta, 1966; Dionisio *et al.*, 2002).

In order to study pCT in a *S. Typhimurium* host background, pCT was transferred by conjugation to a rifampicin resistant SL1344 (L1078) as transformation of pCT into wild-type SL1344 (L354) was unsuccessful on numerous occasions, probably in part due to the size of the plasmid. SL1344 rif^R was chosen as a suitable *S. Typhimurium* SL1344 recipient as the pCT natural host strain C159/11 was found to be susceptible to rifampicin, and the transferrable antibiotic resistance determinants carried on pCT were yet to be determined. When compared to wild-type *S. Typhimurium* SL1344 (L354), *S. Typhimurium* SL1344 rif^R (L1078) was slightly attenuated in its ability to adhere to and invade tissue culture cells. This is consistent with previous literature detailing the effects of rifampicin resistance determinant *rpoB* mutations on host fitness, growth and pathogenicity (Bjorkman *et al.*, 1998; Marianelli *et al.*, 2004; Enne *et al.*, 2005). In the *C. elegans* model however, *S. Typhimurium* SL1344 rif^R was found to kill the nematodes at a faster rate than wild-type SL1344. pCT did not significantly modify the ability of the host bacteria *S. Typhimurium* SL1344 rif^R or *E. coli* 3950 to cause infection in either of the infection models. This corresponds with

the lack of identified virulence genes within the pCT genome (Chapter 3). It may also be predicted that pCT has no effect on expression of host strain virulence factors.

Use of the tissue culture infection model to measure the ability of bacterial strains to cause infection, is simple, standardised and measures the direct pathogen interactions with a single eukaryotic cell type. It also allows exploration of molecular mechanisms of cell adhesion and invasion without much of the complexity of studying infection in a whole animal (Hurley and McCormick, 2003). Data from the *C. elegans* infection model allowed assessment of the ability of bacterial strains to cause infection within a whole organism, therefore complementing data collected from the tissue culture assays. The advantages of studying infection using the *C. elegans* is it is low cost when compared to other animal models, and as bacterial mutants which have attenuated virulence in other animal models also kill nematodes at a slower rate, it is an apt initial model for generation of hypotheses which could be explored further in other animal models to substantiate any findings. (Aballay *et al.*, 2000; Labrousse *et al.*, 2000; Tenor *et al.*, 2004). There are obvious limitations when studying the ability of bacteria to cause infection within a single cells type, or within an animal with no adaptive immune system such as the *C. elegans*, however these model provide a use tool in the study of the ability of bacteria to cause infection.

A relevant difference between the two models is the temperature at which the experiments are conducted. While bacterial cells are incubated within tissue culture cells at 37°C, *C. elegans* are cultured at 25°C. Temperature has been hypothesised to alter expression of bacterial virulence factors, perhaps accounting for differences seen between bacterial strains or pathogenicity models (Lavigne *et al.*, 2006). Therefore if a significant change in host pathogenicity had been identified when pCT

was added to host strains, alternative models to clarify the findings may have been useful next step (Future work).

In the absence of antibiotic pressure, pCT was stably maintained in 100% of bacteria in pure culture in all four bacterial hosts over 96 hours (approx. 70 generations). These data indicate that pCT either has no negative fitness impact on the host cell, and so is able to remain stable within a population, and/or, pCT encodes genes to ensure its persistence. Such genes could encode an addiction and partitioning system.

Another factor that influences plasmid and host fitness is the frequency of plasmid conjugation. The rate of pCT transfer was measured from two donors, 'pCT naive' strain *E. coli* DH5 α and its natural host *E. coli* C159/11 to both *E. coli* and *S. Typhimurium* recipients. The highest rate of transfer from both donors was to recipient *E. coli* J53-2 on a filter, although transfer was lower to the same recipient in liquid. Conversely, transfer to recipient *E. coli* 3950 was higher in liquid than on solid media. As the conjugation frequency of nearly all plasmid incompatibility types can be easily measured on a solid surface (Bradley, 1980), the filter method has become relatively standardised in the literature (Hartskeerl *et al.*, 1985). Plasmid transfer rates in liquid however have been measured using many different protocols (differences in aeration, volume and cell density) therefore it is difficult to compare between studies or to experiments on solid media. Bradley *et al.*, (1980) showed that while plasmids which encode a thick rigid pilus (e.g. IncP/N) can transfer at rates up to 2,000 times higher on agar than in broth, IncI type plasmids (IncI/K) such as pCT, which express both thick (*tra* locus) and thin pili (*pil* locus) could transfer equally well in both conditions. Bradley *et al.* conducted their conjugation experiments over a one

hour period from *E. coli* K12 (nal^S) to K12 (nal^R) and so are not directly comparable with the frequencies in my study (in a 3 hour period to various recipients). However, their data does show the high frequency of transfer in liquid of these IncI plasmids. It may be that pCT transfer into *E. coli* 3950 is higher in liquid than on a filter because *E. coli* 3950, which is derived from pCT's original host C159/11, has adapted to accommodate pCT conjugation. The extensive use of *E. coli* J53 as a conjugation recipient in the laboratory may over time also have inadvertently selected for a capacity for high receipt of plasmid during conjugation on agar as seen in this study and compared to the other strains. My study also highlights that the choice of donor strain and recipient strain has an impact on the conjugation frequency. Therefore experiments with one strain should not be extrapolated to all other bacterial hosts or recipients, even of the same species. The frequency of transfer of pCT from *E. coli* DH5α to *S. Typhimurium* SL1344 rif^R was lower than to *E. coli* recipients J53-2 or DH5α rif^R. These data reflect previous findings that inter-species conjugation occurs at a lower rate than between bacteria of the same species (Hunter *et al.*, 2008). However, pCT transfer from donor *E. coli* C159/11 to *S. Typhimurium* SL1344 rif^R was at a higher rate than from *E. coli* DH5α pCT to *S. Typhimurium* SL1344 rif^R or *E. coli* C159/11 to *E. coli* DH5α rif^R. This finding could have clinical implications, as it implies that pCT can easily transfer to *S. Typhimurium* from its natural host *E. coli* C159/11 on both a solid surface and in liquid, thereby also transferring the *bla*_{CTX-M-14}. While there is no evidence to date that pCT has transferred from an *E. coli* donor to a *S. Typhimurium* recipient in nature, *S. Typhimurium* strains have not been investigated for the presence of pCT-like plasmids, therefore the frequency of transfer between these hosts in nature is currently unknown.

Due to the metabolic burden of DNA replication and pili production, but also the increased risk of bacteriophage infection due to exposure of additional pili, the ability of a plasmid to conjugate at a high frequency has been postulated to have an increased cost to the host bacterial cell (Novotny *et al.*, 1968; Haft *et al.*, 2009). However, Stewart and Levin (1977) argued that a certain level of plasmid transfer is required in order for a plasmid to persist within a population (Stewart and Levin, 1977). Therefore, it seems that natural plasmids achieve a balance between horizontal and vertical transfer (Turner *et al.*, 1998). Some plasmids maximise the rate at which they can conjugate, leading to rapid dissemination, perhaps most beneficial in a heterogeneous population with a high density of potential recipients (Dionisio *et al.*, 2002). These plasmids are likely to confer a larger metabolic cost upon the host, therefore reducing the chances of success of the plasmid-carrying strain over its plasmid free counterparts (Turner *et al.*, 1998). Other plasmids may have a reduced horizontal transfer rate but ensure vertical transmission by conferring a reduced or no fitness burden on the host bacterium (Ferdy and Godelle, 2005). In some cases resulting in a plasmid which loses its ability to conjugate, but is very stable within a particular bacterial strain (Rankin *et al.*, 2011).

In my study, the pCT transfer rate was generally lower from donor *E. coli* C159/11 and to recipient *E. coli* 3950 than to and from 'pCT naive' strains, suggesting that this co-evolved host and plasmid may have adapted to attain a refined (and lower) conjugation frequency and to ameliorate fitness costs.

In the presence of a β -lactam, bacterial cell filamentation has been hypothesised to increase conjugation frequency as the larger average cell length increases contact between cells (Barr *et al.*, 1986). In this study however, no difference was found

between conjugation frequency of pCT with and without the presence of cefotaxime, suggesting the presence of low levels of β -lactams does not significantly increase the spread of plasmid pCT.

In conclusion, pCT was stably maintained in all daughter cells of each host strain over ~70 generations and conferred no fitness cost or benefit on hosts *E. coli* 3950 and *E. coli* DH5 α in the absence of antibiotic selective pressure. pCT did however appear to reduce the competitive ability of *E. coli* J53-2 pCT when co-cultured with its plasmid free parental strain. The pCT transfer rates suggest that liquid mating may be equally, if not more important for the spread of this plasmid as conjugation on a solid surface, particularly within the natural host derivative *E. coli* 3950 and C159/11.

4.7 Further work

In my study, pCT remained stable in all four host strains over ~70 generations, however a recent study of IncA/C plasmids showed that they too remained stable up to 800-1200 generations after which plasmid loss was seen (Subbiah *et al.*, 2011). Therefore, further work could examine the stability of pCT over a greater number of generations to examine the long term effects on plasmid maintenance. The *in vitro* competition experiments could also be carried out over a greater period of time to assess the long term fitness burden or advantage of pCT. This will also allow investigation of whether adaption of the host/plasmid partnership will increase or decrease host fitness when in competition with the plasmid free parental strain. This experiment may be particularly important for host *E. coli* J53-2 where the observed pCT cost upon this host may be ameliorated over time.

The lack of a fitness cost or benefit conferred by pCT to *E. coli* hosts DH5α and 3950 *in vitro* could be explored further to assess how the addition of pCT affects host fitness *in vivo*. A competition assay using the *C. elegans* model was proposed. By feeding the animals on a mixed culture of *S. Typhimurium* (or *E. coli* 3950) with and without pCT at a 1:1 ratio, the proportion of each strain that persists within the gut could be assessed. However, this experiment was not carried out as two problems were highlighted. The first, that pCT would transfer within the bacterial culture, therefore leading to the dominance of pCT carrying bacteria, secondly, pCT7 could not be transferred by conjugation or transformation to *S. Typhimurium* SL1344 rif^R or *E. coli* 3950 and so could not be used in this experiment. Although the culture fed to the *C. elegans* could be controlled so as strains were in a 1:1 ratio, there was also no way to guarantee that the nematodes would consume the same amount of each strain. This would increase the number of nematodes needed in the experiment making it practically impossible to carry out.

Other *in vivo* models to consider for further work on bacterial comparative fitness, but also the infection ability of bacterial hosts and to measure pCT conjugation frequencies *in vivo* are wax moth larvae (*Galleria melonella*), mouse models, and bovine oral infection using calves (Timoney and Linton, 1982; Jarrett and Stephenson, 1990; Savkovic *et al.*, 2005; Lim *et al.*, 2008). All three models have the advantage that a specific infective dose of each bacterial strain can be administered either orally or by injection into the abdomen. Use of the calf model may also be the closest available model to the natural farm environment where pCT was first identified and found to successfully persist (Liebana *et al.*, 2006).

Alternative bacterial species that can act as recipients for pCT conjugation and stably maintain pCT by facilitating plasmid replication could be established to define the pCT host range. Other serovars of *S. enterica* could be explored followed by alternative *Enterobacteriaceae* of clinical relevance such as *Shigella spp.*, *Klebsiella spp.*, *Acinetobacter baumannii* and *Pseudomonas aeruginosa* to aid future screening for pCT in these potential hosts. These experiments alongside further use of the pCT PCR detection test (Chapter 3), will inform whether pCT and the *bla*_{CTX-M-14} gene it encodes are found in other species, in multidrug resistant strains or whether pCT has adapted primarily to an *E. coli* host (Sherley *et al.*, 2003; Norberg *et al.*, 2011).

A fundamental question not addressed within this study is the pCT copy number, and whether this differs within the various host strains tested. As the metabolic load of a plasmid is associated with the copy number, this factor is strongly linked to any fitness burden a plasmid confers upon its host (Loftie-Eaton and Rawlings, 2010). Due to segregation and plasmid maintenance however, a low copy number plasmid may be less likely to persist within a population. Loftie-Eaton and Rawlings (2010) showed that when strains carrying an IncQ plasmid with differing copy numbers were co-cultured *in vitro*, the strain containing the lower copy number plasmid was outcompeted each time. Therefore, how the pCT copy number correlates with fitness is not clear and may depend upon both the host strain and the environmental conditions. To effectively measure a plasmid copy number, many methods are outlined within the literature. These include protocols to quantify gene products such as β -lactamases (Miller and Cohen, 1993), fluorescence proteins such GFP (Wong Ng *et al.*, 2010) and LUX (Coronado *et al.*, 1994); quantification of the plasmid DNA extracted from a defined number of bacterial cells; or comparison of the amount of

plasmid and chromosomal DNA extracted from a culture (Schmidt *et al.*, 1996; Friehs, 2004). Contemporary methods have used quantitative PCR or real time PCR to assess the ratio of amplified plasmid genes to chromosomal genes (Lee *et al.*, 2006; Lee *et al.*, 2006). For example, both studies by Lee *et al.*, (2006) and Lee *et al.*, (2006) quantified the *bla* gene found on pBR322 and a chromosomal gene (16s rDNA and *dxs*) to calculate the copy number of pBR322. For investigation of the pCT copy number in various hosts, a single copy chromosomal gene from each host should be selected and used as the baseline measurement (for example *gyrB* in *E. coli*). Various pCT genes (e.g. *bla*_{CTX-M-14}, *nikB*, *traX*, *pilS*) were all shown to be encoded on pCT in single copies, and any one could be quantified using QPCR allowing the ratio between plasmid and chromosomal DNA to be calculated. The main limitation of this method is that if the DNA extraction favours either plasmid or chromosomal DNA over the other then an accurate copy number estimation will not be obtained. In order to assess this confounder a plasmid of known copy number such as pBR322 (~20 per cell) should be used as a positive control in a quantified number of bacterial cells to assess chromosomal vs. plasmid DNA extraction yields. At this stage, the lack of pCT 'housekeeping' genes or genes known to be expressed at a constant level means that the use of real-time PCR to calculate copy number would be considerably more complicated than QPCR.

Real-time PCR could also be used to determine whether the putative conjugation repressor, *ydbA*, is expressed and whether it actively regulates conjugation. By measuring the expression of *pil* and *tra* locus genes compared to levels of *ydbA* mRNA, hypotheses may be generated about the control of conjugation in different host strains and under different conditions such as on a solid surface and in liquid.

4.8 Key findings

- The addition of pCT to host cells had no significant effect on host phenotype,
 - pCT did not modify the ability of host strains to cause infection,
 - pCT had no significant effect on the generation times of all host strains.
- Non-transferrable pCT (pCT7) did not confer a fitness burden or benefit on *E. coli* DH5α cells in competition with the plasmid free *E. coli* DH5α parent strain.
- When added to a population of potential recipient bacteria, pCT disseminated through cultures of *E. coli* 3950 and *E. coli* DH5α within 96 hours.
- pCT was lost from a culture of *E. coli* J53-2 pCT and *E. coli* J53-2 indicating a cost of the plasmid to this bacterial host strain.
- pCT persisted in 100% of bacterial cells in all four host strains over 96 hours.
- pCT was successfully transferred by conjugation from donors *E. coli* DH5α and *E. coli* C159/11 to *E. coli* and *S. Typhimurium* recipient strains.
- The pCT transfer frequencies were
 - donor and recipient dependent,
 - higher to recipient *E. coli* J53-2 on a filter than in broth,
 - lower to recipient *E. coli* 3950 on a filter than in broth,
 - lower from donor *E. coli* C159/11 than *E. coli* DH5α into the *E. coli* recipient strains,
 - higher from donor *E. coli* C159/11 than *E. coli* DH5α into recipient *S. Typhimurium* SL1344 rif^R.
- The presence of sub-inhibitory concentrations of cefotaxime had no significant effect on the pCT conjugation rate.

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Chapter 5:

The contribution of *bla*_{CTX-M-14} to the biology of pCT

5. The contribution of *bla*_{CTX-M-14} to the biology of pCT

5.1 Background

The obvious benefit to plasmids and their bacterial host strains of acquiring antibiotic resistance genes is that the host is able to survive antibiotic challenge and survive at the detriment of cells which do not possess a resistance gene. Under these conditions, plasmids which carry antibiotic resistance genes are stably maintained within the bacterial population as the resistome is essential for survival. Therefore, the successful spread of antibiotic resistance carrying plasmids has been conventionally attributed to the presence of antibiotic compounds which create a selective pressure and drive both persistence and dissemination of these plasmid vectors (O'Brien, 2002).

In the absence of antibiotic selective pressure, the majority of studies conducted have shown that antibiotic resistance mechanisms carry an initial fitness cost on the host bacterium when compared to their susceptible counterparts (Lenski, 1998; Andersson, 2006; Andersson and Hughes, 2010). For example, mutation in chromosomally encoded *E. coli* streptomycin resistance gene *rpsL* can result in a reduction in the rate of translation and therefore growth, conferring a fitness defect when compared to those cells encoding a wild-type gene (Paulander *et al.*, 2009). The fitness costs associated with antibiotic resistance genes have also been shown to have detrimental effects on the ability of bacterial cells to colonise the upper respiratory tract of rats (Trzcinski *et al.*, 2006), to invade tissue culture cells, and to survive and replicate intra-cellularly (Morosini *et al.*, 2000; Abromaitis *et al.*, 2005). Early studies on antibiotic resistance plasmids, both *in vitro* and *in vivo* (within the

human intestinal tract) indicated that removal of the antibiotic selective pressure resulted in the loss of these plasmids and hence their antibiotic resistance genes from the bacterial strains (Anderson, 1974; Andersson and Levin, 1999). This was due to the inherent fitness costs of these plasmids which allowed susceptible strains to outcompete the resistant ones. Contrary to these findings, Lenski *et al.*, (1994) showed that the fitness benefit conferred by plasmid pACYC184 to its *E. coli* host after a period of co-evolution was reversed when the tetracycline resistance gene (*tetC*) was removed or inactivated. These data suggest an additional role of this antibiotic resistance gene in the plasmid and host interaction (Lenski *et al.*, 1994). Therefore, the contribution of antibiotic resistance genes to the evolutionary success and dissemination of large natural plasmids is still largely undetermined.

Despite strategies to removal or reduce antibiotic selective pressure by limitation of antibiotic usage through stewardship or ‘cycling’ and therefore decrease the number of resistant strains, the majority of studies have shown that the resistance determinants remain within the bacterial populations (Kollef, 2006). This suggests that interactions between the antibiotic resistance genes, their vectors, the host bacteria and the real world are complex.

5.2 Hypotheses and Aims

The presence of the antibiotic resistance gene *bla*_{CTX-M-14} plays an important role in the evolutionary success of plasmid pCT as it confers either a neutral or beneficial effect on pCT or the host strain, allowing the plasmid to persist both in the presence and absence of antibiotic pressure.

The aims of this work were to:

- Develop a method for the targeted inactivation of plasmid encoded genes,

- Use this method to inactivate *bla*_{CTX-M-14} on pCT by insertion of a kanamycin resistance gene, *aph*, and green fluorescent protein gene, *gfp*,
- Measure the biological effects of inactivation of *bla*_{CTX-M-14} on pCT and its bacterial host strains.
- Assess the effect of removal of the *bla*_{CTX-M-14} upstream insertion element *ISEcp1* has on host susceptibility to β -lactam antibiotics cefotaxime and ceftiofur.

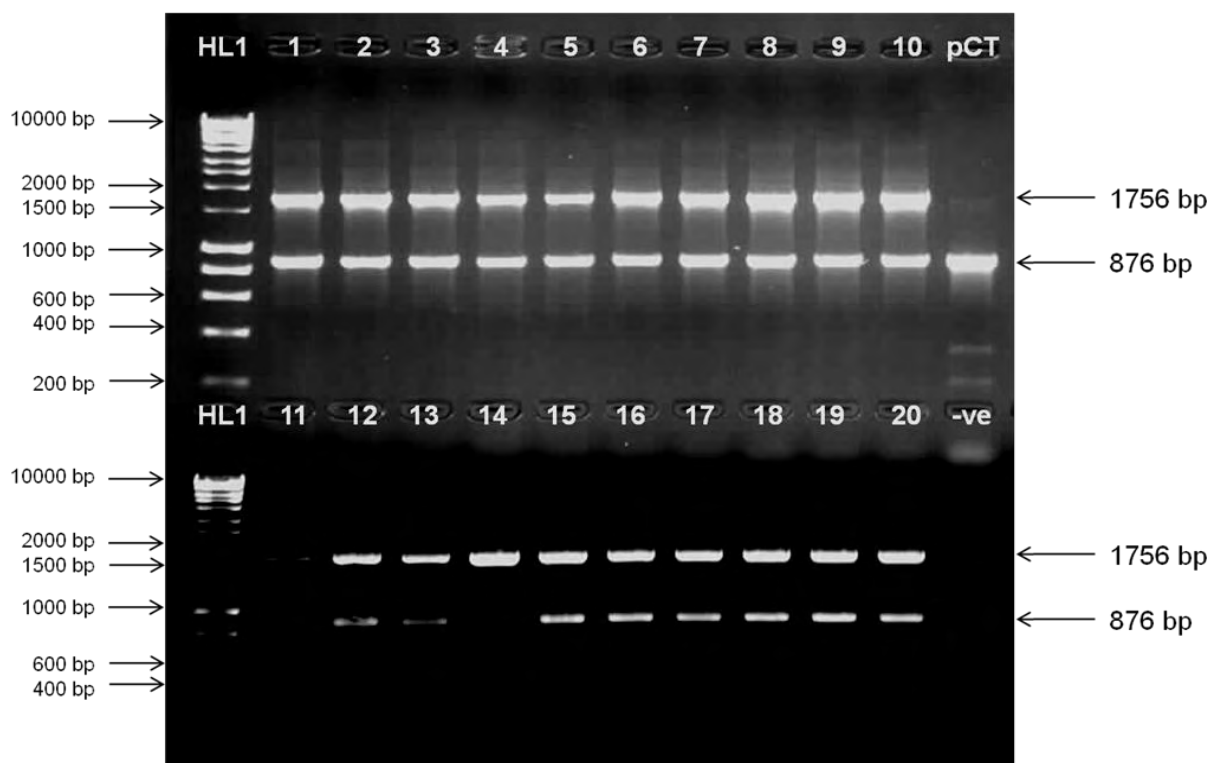
5.3 Development of a method to inactivate pCT *bla*_{CTX-M-14}

Conventional methods to inactivate genes on plasmids have previously used random insertion of transposons followed by screening to identify the disrupted gene of interest. Site specific methods have been devised for the inactivation of bacterial chromosomal genes using homologous recombination (Datsenko and Wanner, 2000), however, targeted approaches to inactivating specific plasmid genes have not been well documented in the literature. Initially, a protocol based upon methods for chromosomal gene inactivation outlined by Datsenko and Wanner (2000) were followed. Briefly, recombination of a resistance marker amplified by PCR with regions of homology to *bla*_{CTX-M-14} at either end of the amplicon, required the transformation of plasmid pKD46, encoding a lambda-red recombinase, into a pCT containing bacterial strain. Problems with co-location and maintenance of both pKD46 and pCT within a single cell led to the development of a novel method which used a chromosomally based lambda-red recombinase, removing the need for a double transformation and maintenance of both plasmids. *E. coli* strain SW102 (l759) encoding the chromosomal lambda-red recombinase was kindly donated by Dr Howard Bayliss (University of Cambridge).

5.4 Inactivation of pCT *bla*_{CTX-M-14} by insertion of *aph*

PCR primers were designed with 20 bp homology to L109 (previously recombined with *aph* amplified from pKD4) in order to amplify the kanamycin resistance gene *aph*, and 40 bp of homology to the outer regions of *bla*_{CTX-M-14} (Table 2.7, primer nos. 823/824). Homologous recombination of this PCR product with pCT allowed the removal of 606 bp of *bla*_{CTX-M-14} including the sequence encoding the enzyme's active site, and its replacement with *aph*. Initially, pCT DNA and the PCR amplicon encoding *aph* were co-transformed together by electroporating both plasmid and PCR amplicon into freshly made competent *E. coli* SW102 cells at the same time. The low frequency of pCT transformation in addition to the low frequency of recombination meant no recombinant plasmids were obtained. Therefore, the method was modified to include a two step process where pCT was transformed and selected in *E. coli* SW102 first (SW102 pCT), followed by subsequent electroporation of the PCR construct. This allowed the establishment of pCT in *E. coli* SW102 and maximised the chance of recombination. Using this new strain, *E. coli* SW102 pCT (I879), twenty colonies grew on kanamycin containing agar (50 µg/ml), indicating successful recombination of pCT and *aph* had taken place. Each colony was sub-cultured onto fresh agar containing kanamycin and cell lysates made for each one. Lysates were used as the DNA template in a PCR reaction with primers designed upstream and downstream of the recombination region (Table 2.7, primer no. 819/820). With wild-type pCT DNA it was expected that these primers would yield amplicons of 876 bp. However if the *aph* gene had been successfully recombined, an amplicon of 1764 bp would be obtained. PCR resulted in both 876 bp and 1765 bp amplicons from all twenty lysates (Figure 5.1), therefore indicating the presence of both wild-type (pCT) and a recombinant plasmid (pCT *bla*_{CTX-M-14::aph}).

Figure 5.1. PCR amplimers from SW102 colonies containing putative mutated pCT (*bla*_{CTX-M-14}::*aph*)



| Feature | Description |
|---------|---|
| HL1 | Hyperladder 1 (Bioline) |
| 1-20 | <i>E. coli</i> SW102 candidate colonies putatively containing pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) |
| pCT | Wild-type control |
| -ve | Negative water control |

Twenty colonies containing putative mutated pCT after recombination were selected and used as the DNA template in a PCR reaction with primers designed upstream and downstream of the recombination region. In each reaction the presence of a mutated plasmid was indicated by the presence of a 1756 bp amplimer, however the presence of a 875 bp amplimer also suggested the presence of wild-type pCT in each lysate. Amplimers were visualised using electrophoresis on a 1% agarose gel.

Plasmid DNA was harvested from colony 14 as visualisation of PCR amplimers after electrophoresis on an agarose gel indicated the presence of relatively less wild-type pCT within this colony (Figure 5.1), and the plasmid DNA transformed again into *E. coli* DH5 α . This removed the possibility of further recombination from residual λ -red recombinase activity in *E. coli* SW102 cells. Both wild-type pCT and mutant plasmid were still detected using PCR within the *E. coli* DH5 α colonies selected on kanamycin. Therefore, a colony was selected and 'cycled' on LB agar containing 50 μ g/ml kanamycin so that upon segregation of each bacterial cell, the *aph* containing plasmid would be selected and those bacterial cells containing wild-type pCT only would be inhibited. Each day, colonies were chosen at random and lysates used in a PCR to amplify the *bla*_{CTX-M-14} region. After six days this PCR only amplified fragments of 1764 bp in size in all colonies tested, and when replica plated onto fresh agar, colonies were also unable to grow in the presence of 8 μ g/ml of cefotaxime. Plasmid DNA was harvested from this strain and transformed into *E. coli* DH5 α cells to remove any effects on the host from serial passaging. A colony was chosen, checked for the presence of pCT *bla*_{CTX-M-14}::*aph* only, and named *E. coli* DH5 α pCT2 (I778). DNA sequencing of PCR amplimers from this colony showed the outer coding regions of *bla*_{CTX-M-14}, the recombining sites and the *aph* gene in the expected positions, verifying successful recombination (Figure 5.2). pCT2 was also conjugated into recipient *E. coli* strains J53-2 (I847), *E. coli* 3950 (I823) and *S. Typhimurium* SL1344 rif^R (L1078) creating strains *E. coli* J53-2 pCT2 (I856), *E. coli* 3950 pCT2 (I835) and *S. Typhimurium* SL1344 rif^R pCT2 (L1244).

To further confirm the inactivation of *bla*_{CTX-M-14} in pCT2, the chromogenic β -lactam nitrocefin was used to detect the production of β -lactamase. Lysates of *E. coli* C159/11, *E. coli* DH5 α pCT and *S. Typhimurium* SL1344 rif^R pCT (L1079) containing

Figure 5.2. DNA sequence showing the successful insertion of the *aph* gene into *bla*_{CTX-M-14} on pCT

```

GCTGGGCAGCGCGCCGC TTTATGCGCAGACGAGTGGGTGCAGCAAAAGCTGGCGGC GTGTAGGCTGG
AGCTGCTTC GAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCAAGATCCCCCA
CGCTGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCA
GAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAA
AGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGGACAGCA
AGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAAAGTAACTGGAT
GGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGAT
CGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCCTCCGGCCGCTTGGGTGGAGAGGCTATTC
GGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGG
GCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGC
GGCTATCGTGGCTGGCCACGACGGGCGTTCCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGA
AGGGACTGGCTGCTATTGGGCGAAGTGCCGGGGCAGGATCTCCTGTCACTCACCTTGCTCCTGCCGA
GAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCG
ACCACCAAGCGAAACATCGCATCGAGCGAGCACGTA CTGGATGGAAGCCGGTCTTGTCGATCAGGAT
GATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAC TGTTCGCCAGGCTCAAGGCGCGCATGCC
CGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTGGAAAATGGCC
GCTTTTCTGGATTTCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCAGGACATCGCGTTGGCT
ACCCGTGATATTGCTGAAGAGCTTGCGGCGAATGGGCTGACCGCTTCCTCGTGCTTTACGGTATCGC
CGCTCCCGATTTCGACGCGCATCGCCCTCTATCGCCTTCTTGACGAGTTCTTCTGAGCGGGACTCTGGG
GTTCGAAATGACCGACCAAGCGACGCCAACCTGCCATCACGAGATTTTCGATTCCACCGCCGCCTTCT
ATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTC
ATGCTGGAGTTCTTCGCCCACCCAGCTTCAAAGCGCTCTGAAGTTCCATACTTTCTAGAGAATAG
GAACTTCGGAATAGGAACTAAGGAGGATATTCAT ATGGACCATGGCTAATTCCC CGACTACGGCACCA
CCAATGATATTGCGGTGATCTGG CCGCAGGGTCGTGCGCCGCTGGTTCTGGTGACCTATTTTACCCA

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The kanamycin resistance gene *aph* was recombined with pCT gene *bla*_{CTX-M-14}.

PCR amplification across this genomic region and sequencing of the amplicon generated was used to confirm successful recombination.

Red text, DNA sequence homologous to the *bla*_{CTX-M-14} gene,

Black text, DNA sequence homologous to the *aph* gene region in L109,

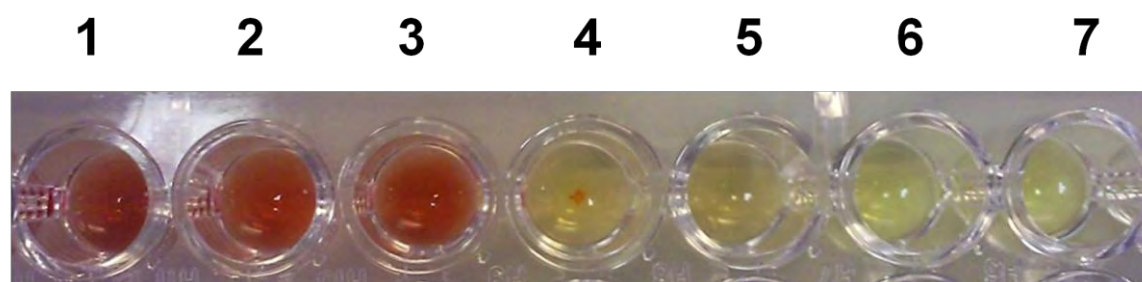
Green highlighted text, DNA region where homologous recombination has taken place.

wild-type pCT turned nitrocefin red, demonstrating the production of a β -lactamase (Figure 5.3). Host strains *E. coli* DH5 α (1825) and *S. Typhimurium* SL1344 rif^R (L1078) without plasmid did not produce a colour change with nitrocefin. Both strains containing pCT2 were also unable to produce β -lactamase, failing to cause a colour change of nitrocefin from yellow to red.

5.5 Inactivation of pCT *bla*_{CTX-M-14} with a *gfp*

In order to mark pCT with a constitutively expressed *gfp* for rapid detection and tracking of the plasmid, the *gfpmut2* gene was inserted into *bla*_{CTX-M-14} using the same method as previously described in Section 5.4. Plasmid pUA66*pacpP* encoding adjacent *aph* and *gfp* genes was fully digested using restriction enzymes to prevent carry-over of intact plasmid into the PCR mixture, and reducing the selection of colonies which carried pUA66*pacpP* instead of a modified pCT. Digested plasmid DNA was the template for amplification of *aph-gfpmut2* with primers designed with 20 bp homology to upstream and downstream of the *aph-gfpmut2* region on pUA66*pacpP* and with the same 40 bp homology to *bla*_{CTX-M-14} as previously described (Table 2.7, primer nos. 1038/1039). PCR yielded a 2438 bp fragment. Recombination using amplified *aph-gfpmut2* was technically more difficult than with *aph* alone, probably due to the increased size of the PCR fragment (2,361 bp vs 1,256 bp), therefore a higher yield of the PCR amplimer (*aph-gfpmut2*) (0.2 ng/ml of DNA) was required. Transformation of amplimers into freshly made competent and activated *E. coli* SW102 pCT (1879) cells produced seven kanamycin resistant colonies which fluoresced under excitation with light at 395 nm. All seven colonies were sub-cultured and their plasmid DNA harvested for PCR. Using primers external to the recombination region (Table 2.7, primer nos. 819/820), wild-type pCT

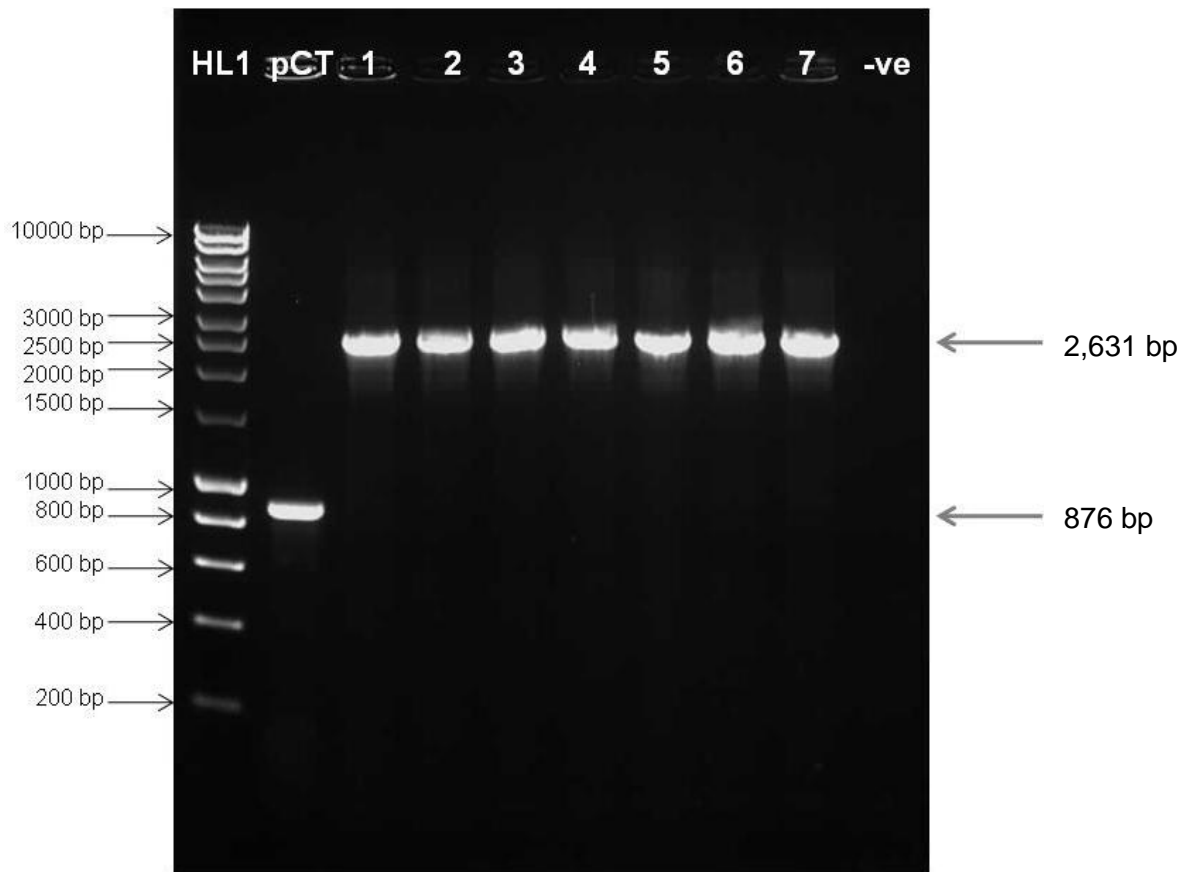
Figure 5.3. Nitrocefin test for β -lactamase production



| Well No | Laboratory number | Strain (description) | β -lactamase activity |
|---------|-------------------|---|-----------------------------|
| 1 | I753 | <i>E. coli</i> C159/11 | + |
| 2 | I755 | <i>E. coli</i> DH5 α pCT | + |
| 3 | L1079 | <i>S. Typhimurium</i> SL1344 rif ^R pCT | + |
| 4 | I778 | <i>E. coli</i> DH5 α pCT2 (<i>bla</i> _{CTX-M-14::aph}) | - |
| 5 | L1244 | <i>S. Typhimurium</i> SL1344 rif ^R pCT2 (<i>bla</i> _{CTX-M-14::aph}) | - |
| 6 | I825 | <i>E. coli</i> DH5 α | - |
| 7 | L1078 | <i>S. Typhimurium</i> SL1344 rif ^R | - |

β -lactamase production in each strain was investigated using synthetic indicator nitrocefin. Production of β -lactamase enzyme was indicated by a colour change from yellow (-) to red (+) <5 minutes after the addition of nitrocefin. Non β -lactamase producers failed to produce a colour change and remained yellow (-).

Figure 5.4. PCR amplimers from *E. coli* SW102 colonies containing putative mutated pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) separated by electrophoresis



| Feature | Description |
|---------|---|
| HL1 | Hyperladder 1 |
| 1-7 | SW102 candidate colonies putatively containing pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) |
| pCT | Positive control (wild-type) |
| -ve | Negative control (water) |

Successful recombination of the *aph-gfpmut2* PCR construct with pCT *bla*_{CTX-M-14} was indicated by the amplification of a large 2,631 bp PCR amplimer from putative mutant plasmids (Candidate colonies 1-7) when compared to a wild-type product of 876 bp.

Amplimers were visualised using electrophoresis on a 1% agarose gel.

generated a fragment of 876 bp compared with putative inactivated plasmids, all of which produced amplicons of 2631 bp only (Figure 5.4). The PCR amplicon yielded from colony 1 was sequenced and showed the outer regions of *bla*_{CTX-M-14}, the recombination sites and internal *aph* and *gfpmut2* genes in the expected positions (Appendix 12). Therefore, plasmid DNA from colony 1 was extracted and transformed into *E. coli* DH5 α . A colony from this transformation was stored and named *E. coli* DH5 α pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) (I826). pCT2b was also transferred by conjugation to recipient strains *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R creating strains *E. coli* J53-2 pCT2b (I857), *E. coli* 3950 pCT2b (I836) and *S. Typhimurium* SL1344 rif^R pCT2b (L1235).

5.6 Removal of *bla*_{CTX-M-14} upstream insertion sequence *ISEcp1*

It has been suggested that in the absence of a promoter region directly upstream of *bla*_{CTX-M}, that a promoter sequence situated in the adjacent *ISEcp1* element may allow expression or up-regulation of the β -lactamase (Poirel *et al.*, 2003). To investigate the influence of *ISEcp1* on pCT, 1008 bp of the *ISEcp1* region was removed using the recombination technique described (Section 5.4), and replaced with the kanamycin resistance gene, *aph*. Due to primer design constraints only two of the three putatively identified promoter regions could be removed using this method. The altered plasmid was verified by PCR and sequencing (Appendix 13) and transformed into *E. coli* DH5 α ; this strain was named *E. coli* DH5 α pCT6 (*ISEcp1*::*aph*) (I830). pCT6 was also transferred by conjugation into *E. coli* J53-2 (I847), *E. coli* 3950 (I823) and *S. Typhimurium* SL1344 rif^R (L1078), creating strains *E. coli* J53-2 pCT6 (I861); *E. coli* 3950 pCT6 (I838) and *S. Typhimurium* SL1344 rif^R pCT6 (L1245).

5.7 Effects on pCT plasmid biology of inactivating *bla*_{CTX-M-14} and *ISEcp1*

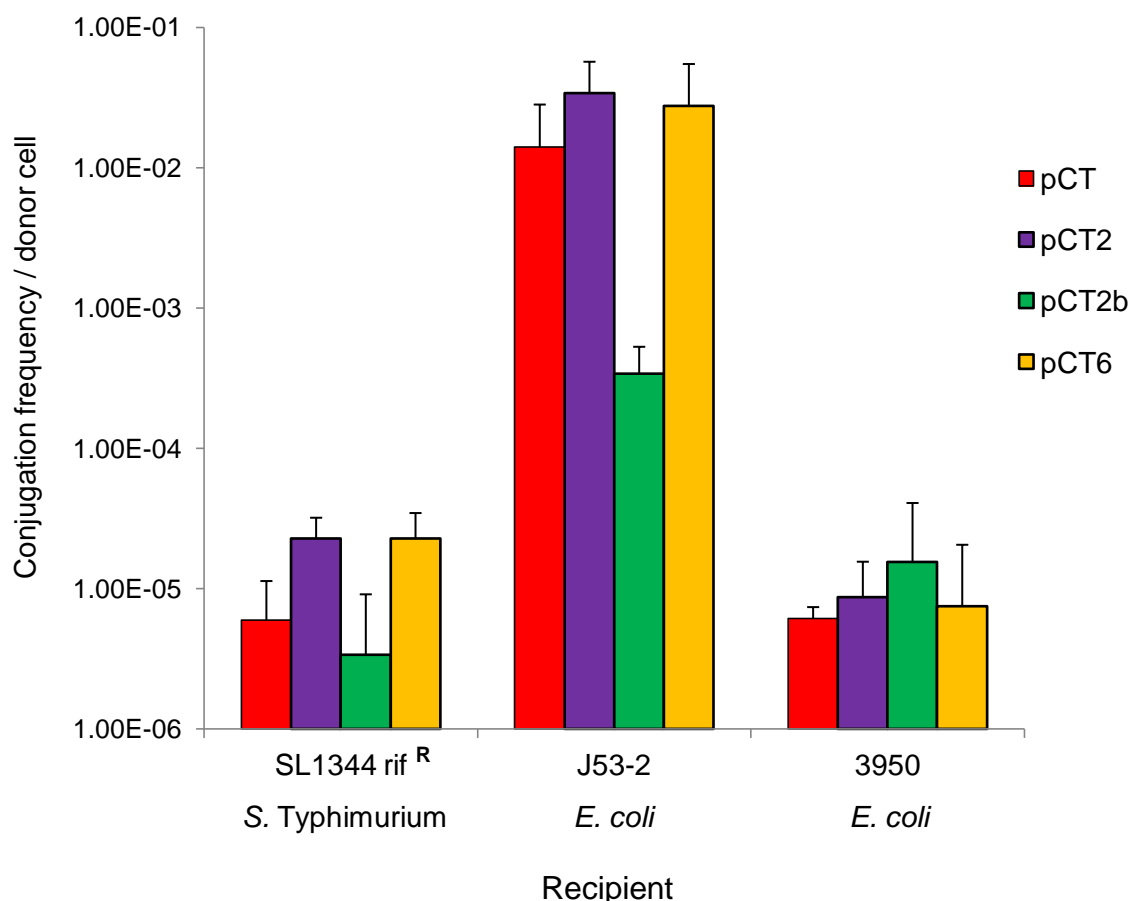
5.7.1 Stability of plasmids pCT2, pCT2b and pCT6 in host cells

Wild-type pCT was stably maintained in 100% of bacterial cells without the presence of antibiotic selection over ~70 generations (Chapter 4). To assess whether inactivation of the *bla*_{CTX-M-14} gene and removal of *ISEcp1* affected the maintenance and persistence of pCT, *E. coli* DH5 α , *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R containing plasmids pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfp*) and pCT6 (*ISEcp1*::*aph*) were grown in isogenic cultures over 96 hours in LB broth without antibiotics with passage into fresh broth after 12 hours, and subsequently every 24 hours. Over the 96 hours all three plasmids were stably maintained within 100% of the four bacterial host strains, showing that inactivation of the *bla*_{CTX-M-14} had no detrimental effect on pCT persistence under these conditions.

5.7.2 Conjugative transfer of pCT2, pCT2b and pCT6 compared to pCT

The conjugation frequency of each mutant plasmid was measured from donor *E. coli* DH5 α cells to recipient bacteria over a three hour period at 37⁰C both on solid (Figure 5.5, Appendix 14) and in liquid media (Figure 5.6, Appendix 15). Inactivation of *bla*_{CTX-M-14} (pCT2) and removal of *ISEcp1* (pCT6) had no significant effect on the frequency of pCT conjugation to any of the recipient hosts, in liquid or a solid surface. The addition of *gfpmut2* (pCT2b) reduced the frequency of pCT transfer to recipient *E. coli* J53-2, but did not affect conjugation into the other recipient strains (*E. coli* 3950 and *S. Typhimurium* SL1344 rif^R). Therefore, these data suggest that inactivation of *bla*_{CTX-M-14} had no affect on the ability of pCT to conjugate to the recipient strains tested in the absence of antibiotic pressure.

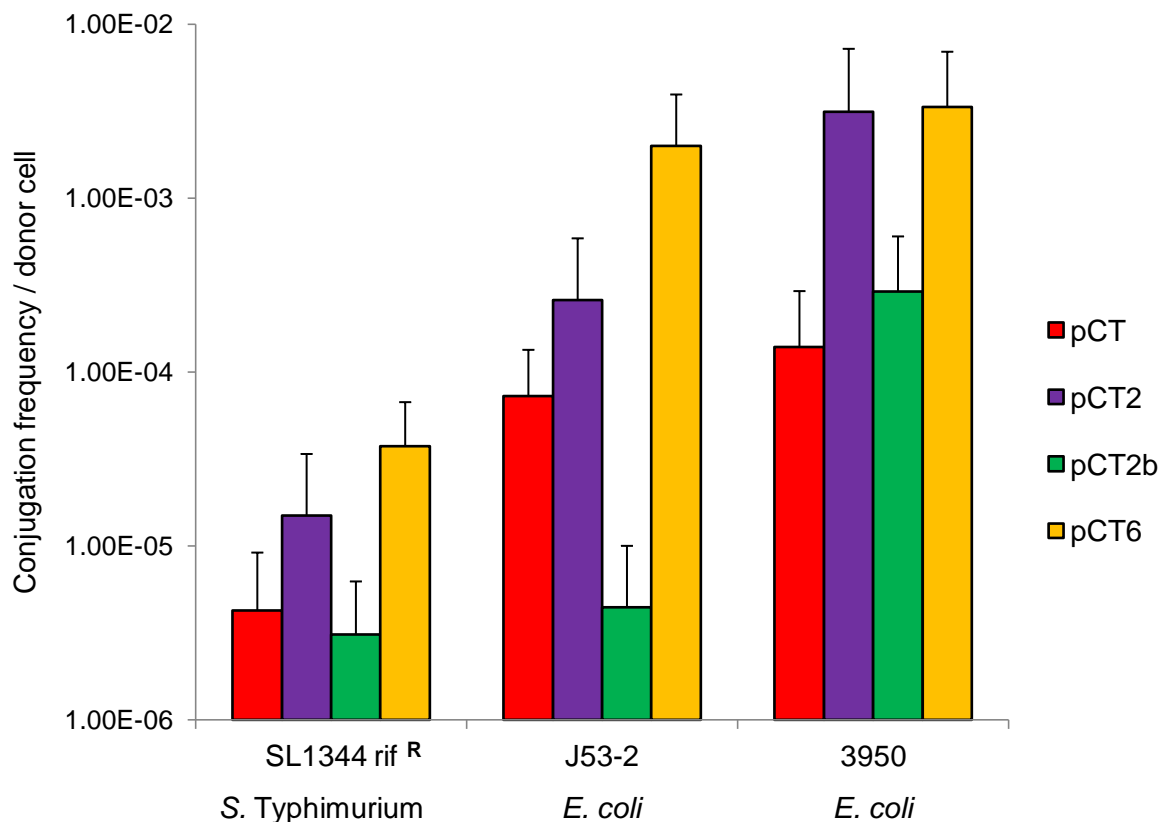
Figure 5.5. Conjugation frequencies of pCT and *bla*_{CTX-M-14} mutants pCT2, pCT2b and pCT6 on solid media to various recipients



The conjugation frequency of pCT2 (*bla*_{CTX-M-14}::*aph*)(purple), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*)(green) and pCT6 (*ISEcp1*::*aph*)(yellow) were compared to the conjugation frequency of wild-type pCT (red) from donor bacterial strain *E. coli* DH5α to recipient strains *S. Typhimurium* SL1344 rif^R, *E. coli* J53-2 and *E. coli* 3950 on a filter.

Conjugation frequencies were calculated from the mean of at least three separate experiments (Appendix 14). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant are represented with an asterix.

Figure 5.6. Conjugation frequencies of pCT and *bla*_{CTX-M-14} mutants pCT2, pCT2b and pCT6 in liquid media to various recipients



The conjugation frequency of pCT2 (*bla*_{CTX-M-14}::*aph*)(purple), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*)(green) and pCT6 (*ISEcp1*::*aph*)(yellow) were compared to the conjugation frequency of wild-type pCT (red) from donor bacterial strain *E. coli* DH5α to recipient strains *S. Typhimurium* SL1344 rif^R, *E. coli* J53-2 and *E. coli* 3950 in liquid media.

Conjugation frequencies were calculated from the mean of at least three separate experiments (Appendix 15). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are represented with an asterisk.

5.8 Effects of pCT2, pCT2b and pCT6 upon the bacterial host strain compared to wild-type pCT

5.8.1 Susceptibility to cefotaxime and ceftiofur

The MIC of cefotaxime and ceftiofur for each host strain containing plasmids pCT2, pCT2b and pCT6 were compared to that of host strains containing wild-type pCT. pCT was shown to confer resistance to both cefotaxime and ceftiofur when transferred to a new bacterial host therefore these drugs were selected to represent commonly used clinical and veterinary third generation cephalosporins respectively. In all hosts, the inactivation of *bla*_{CTX-M-14} (pCT2 and pCT2b) reduced the MIC of both drugs back to that for the corresponding strain with no pCT (Table 5.1). This confirmed that this gene conferred resistance to cefotaxime. Inactivation of the *bla*_{CTX-M-14} upstream element *ISEcp1* had no effect on host susceptibility to cefotaxime or ceftiofur as the MIC of these agents was found to be the same for all host strains containing pCT and pCT6 (Table 5.1).

5.8.2 Growth rate of host bacteria containing pCT2, pCT2b and pCT6

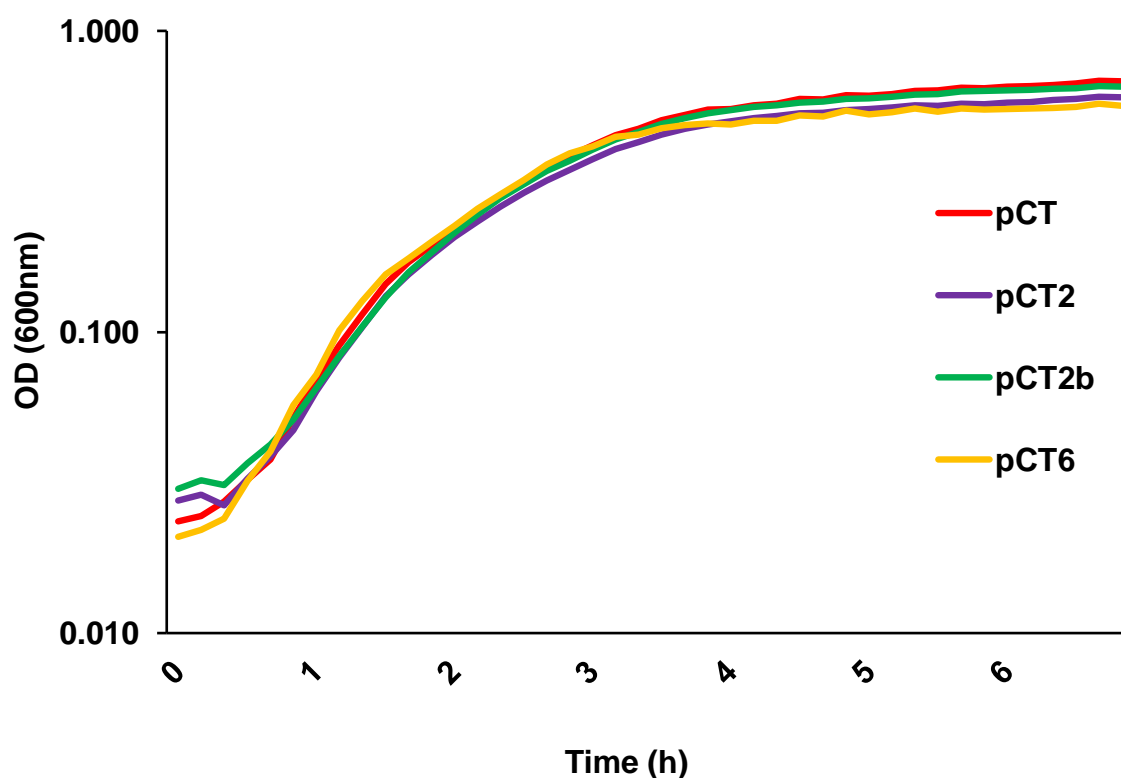
To establish whether *bla*_{CTX-M-14} modification had an effect on host strain growth, the growth kinetics of host strains containing pCT2, pCT2b and pCT6 were compared to those containing pCT. The optical densities (600 nm) of three biological and four technical repeats for each strain, were measured automatically by a FLUOstar OPTIMA (BMG Labtech, UK) every ten minutes for 16 hours. From these data the generation times during mid-logarithmic phase were calculated and compared. The inactivation of the pCT *bla*_{CTX-M-14} by the addition of *aph* or *aph-gfpmut2* (pCT2 and pCT2b) and the removal of pCT *ISEcp1* (pCT6) had no significant effect on the generation times of *E. coli* hosts 3950 (Figure 5.7, Appendix 16) or *E. coli* J53-2 (Figure 5.8, Appendix 17). The generation times of host *E. coli* DH5α containing

Table 5.1. Minimum inhibitory concentrations of two β -lactam antibiotics for hosts containing pCT and *bla*_{CTX-M-14} modified plasmids pCT2, pCT2b and pCT6

| Laboratory number | Strain | MIC of cefotaxime ($\mu\text{g/ml}$) | MIC of ceftiofur ($\mu\text{g/ml}$) |
|-------------------|--|--|---------------------------------------|
| I753 | C159/11 | 32 | 64 |
| I825 | DH5 α | 0.03 | 0.25 |
| I755 | DH5 α pCT | 16 | 32 |
| I778 | DH5 α pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 0.03 | 0.5 |
| I826 | DH5 α pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | 0.03 | 0.25 |
| I830 | DH5 α pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 16 | 32 |
| I847 | J53-2 | 0.03 | 0.5 |
| I834 | J53-2 pCT | 16 | 32 |
| I856 | J53-2 pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 0.03 | 0.5 |
| I857 | J53-2 pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | 0.03 | 0.5 |
| I861 | J53-2 pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 16 | 32 |
| I823 | 3950 | 0.03 | 0.5 |
| I855 | 3950 pCT | 16 | 64 |
| I835 | 3950 pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 0.03 | 0.5 |
| I836 | 3950 pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | 0.03 | 0.5 |
| I838 | 3950 pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 16 | 64 |
| L1078 | SL1344 rif ^R | 0.06 | 1 |
| L1079 | SL1344 rif ^R pCT | 64 | 64 |
| L1244 | SL1344 rif ^R pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 0.03 | 1 |
| L1235 | SL1344 rif ^R pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfpmut2</i>) | 0.03 | 1 |
| L1245 | SL1344 rif ^R pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 64 | 64 |

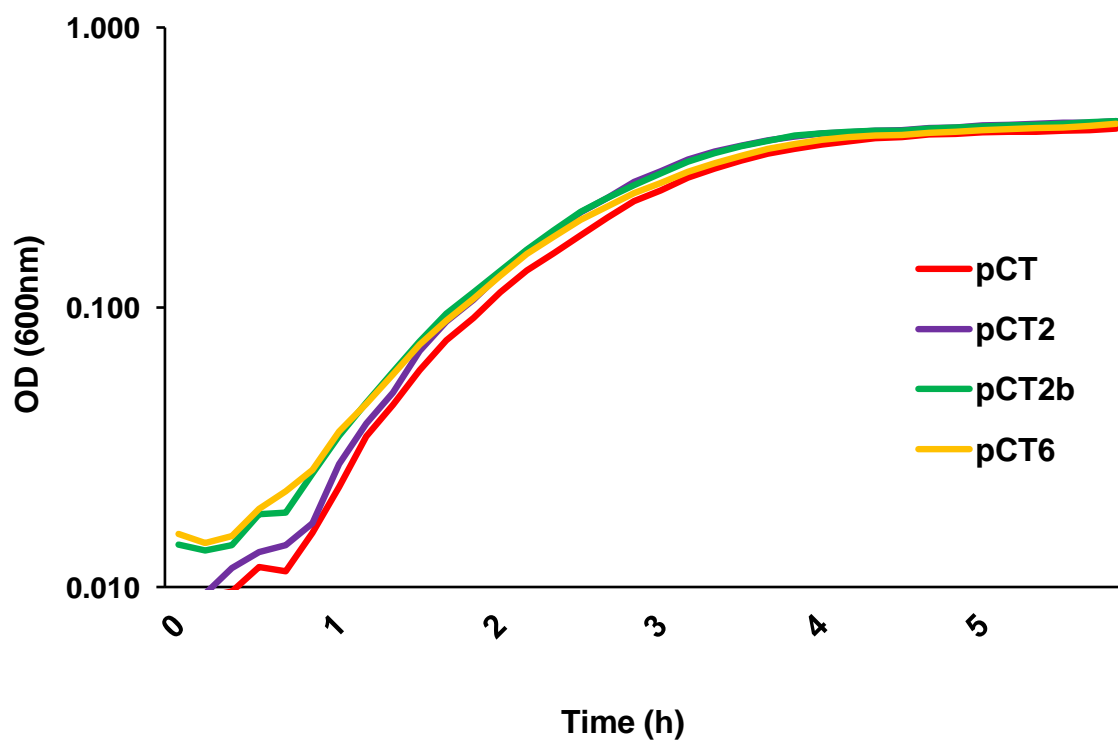
MICs were taken as the modal value of at least three separate experiments with an acceptable error of one doubling dilution. Bold text indicated a change in MIC due to inactivation of *bla*_{CTX-M-14}.

Figure 5.7. Growth kinetics of *E. coli* 3950 containing plasmids pCT2, pCT2b and pCT6 compared to *E. coli* 3950 pCT



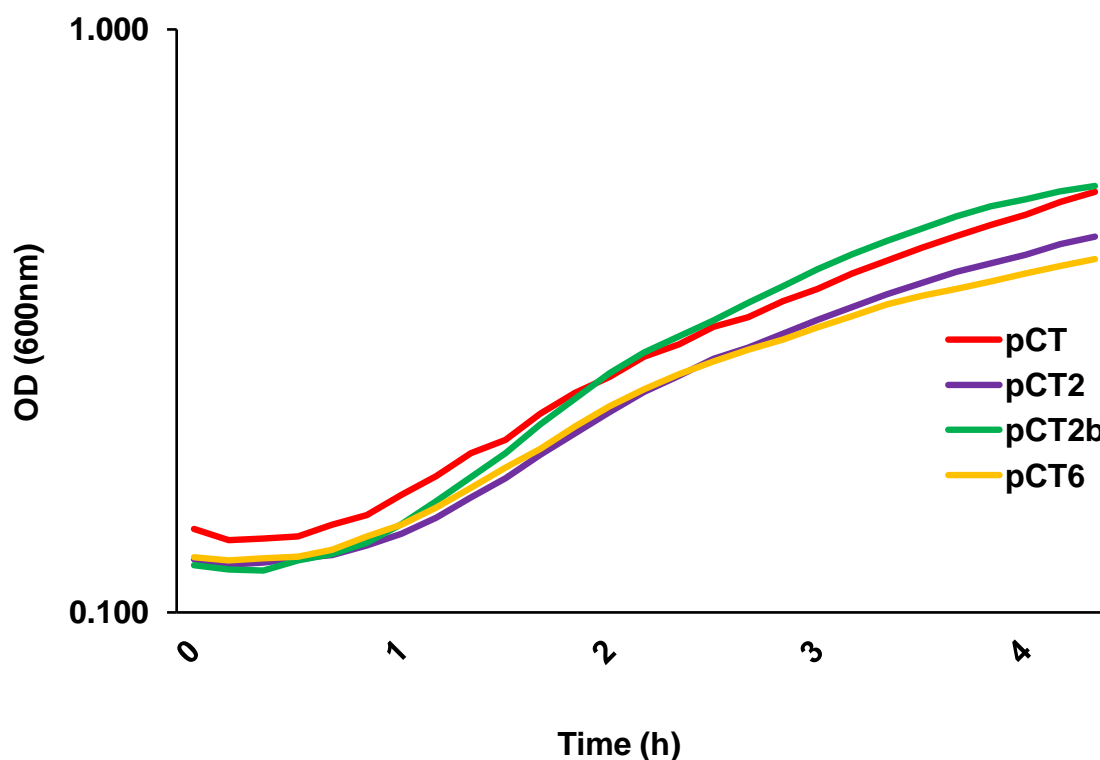
The growth kinetics of bacterial host strain *E. coli* 3950 containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Appendix 16).

Figure 5.8. Growth kinetics of *E. coli* J53-2 containing plasmids pCT2, pCT2b and pCT6 compared to *E. coli* J53-2 pCT



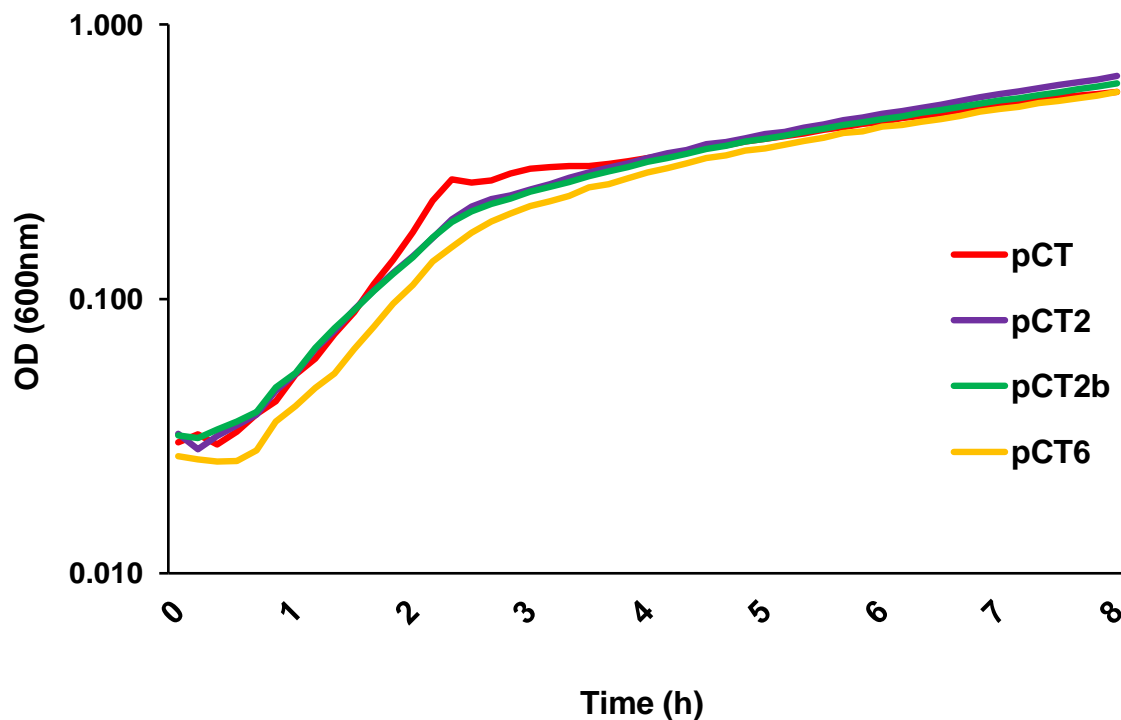
The growth kinetics of bacterial host strain *E. coli* J53-2 containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Appendix 17).

Figure 5.9. Growth kinetics of *E. coli* DH5 α containing plasmids pCT2, pCT2b and pCT6 compared to *E. coli* DH5 α pCT



The growth kinetics of bacterial host strain *E. coli* DH5 α containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Appendix 18). The generation time during mid-logarithmic phase of *E. coli* DH5 α pCT2b was found to be statistically significantly different from that of *E. coli* DH5 α pCT ($P = 0.02$) when a P value of <0.05 was returned using the Student's T test (Appendix 18).

Figure 5.10. Growth kinetics of *S. Typhimurium* rif^R containing plasmids pCT2, pCT2b and pCT6 compared to *S. Typhimurium* rif^R SL1344 pCT



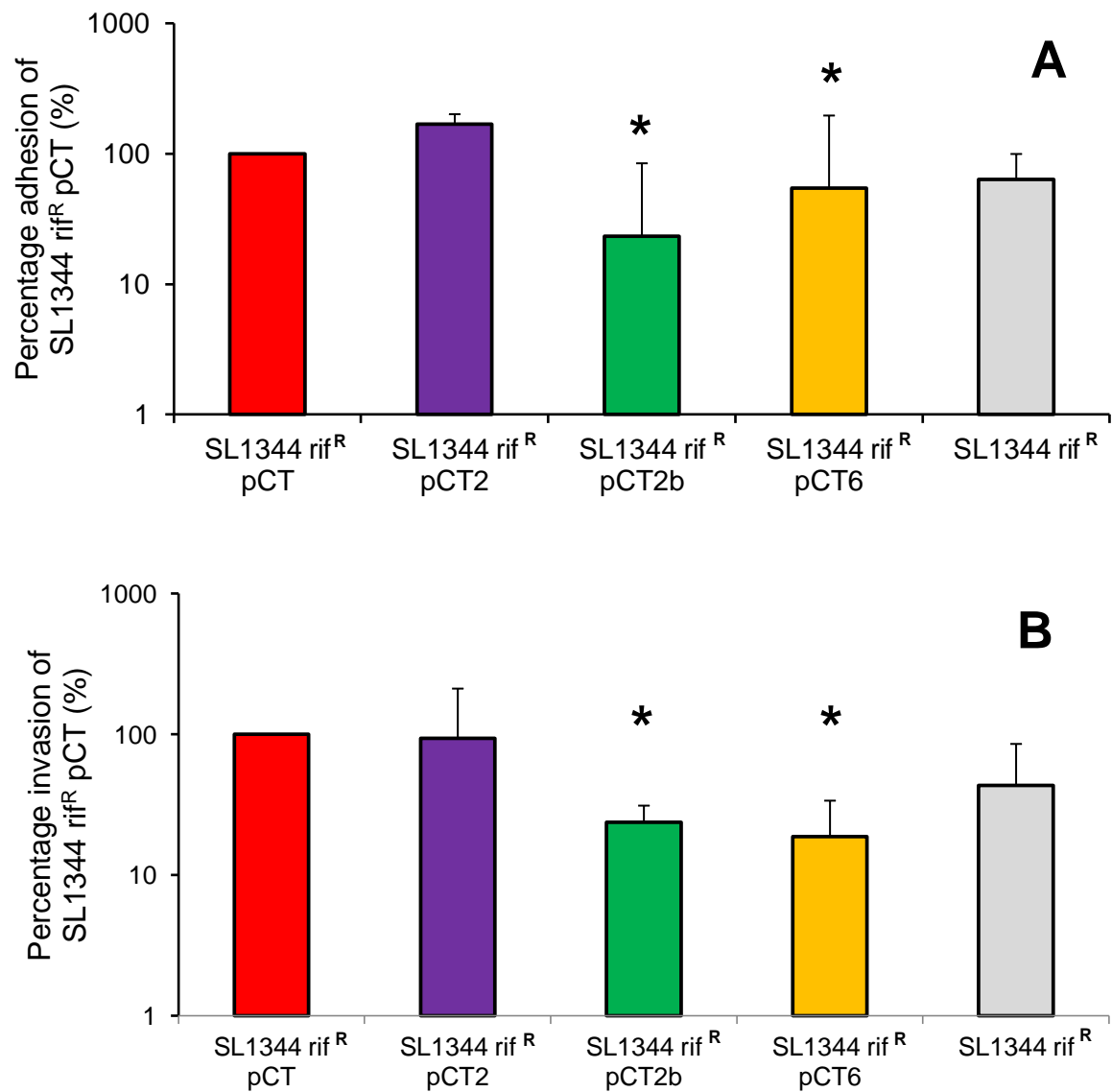
The growth kinetics of bacterial host strain *S. Typhimurium* SL1344 rif^R containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Appendix 19). The generation time during mid-logarithmic phase of *S. Typhimurium* SL1344 rif^R pCT6 was found to be statistically significantly different from that of *E. coli* *S. Typhimurium* SL1344 rif^R pCT ($P = 0.036$) when a P value of <0.05 was returned using the Student's T test (Appendix 19).

pCT2 and pCT6 were unchanged when compared to *E. coli* DH5 α pCT, however *E. coli* DH5 α pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) had a significantly decreased generation time during mid-logarithmic phase (Figure 5.9, Appendix 18). In *S. Typhimurium* host SL1344 rif^R (Figure 5.10), inactivation of *bla*_{CTX-M-14} (pCT2 and pCT2b) had no effect on generation times, however, the removal of *ISEcp1* (pCT6) resulted in a statistically significant increase ($P = 0.036$) in the generation time of during mid-logarithmic phase (Figure 5.10, Appendix 19).

5.8.3 The effect of pCT2, pCT2b and pCT6 on the ability of bacterial host strains to cause infection

The effect of inactivating pCT *bla*_{CTX-M-14} and removal of *ISEcp1* on the ability of host strains to cause infection when compared to hosts containing wild-type pCT were investigated using tissue culture and *C. elegans* models of infection. Inactivation of *bla*_{CTX-M-14} by insertion of *aph* alone (pCT2) had no significant effect on the ability of *S. Typhimurium* SL1344 rif^R to adhere to, or invade intestinal cells (Figure 5.11, Appendix 20); likewise there was no significant difference in the rate of *C. elegans* killing (Figure 5.12, Appendix 21). Inactivation of *bla*_{CTX-M-14} with *aph* and *gfpmut2* (pCT2b) also had no effect on the host strains ability to kill *C. elegans* (Figure 5.12) however did reduce the number of *S. Typhimurium* SL1344 rif^R able to adhere to and invade INT-407 cells when compared to *S. Typhimurium* SL1344 rif^R pCT (Figure 5.11B). Lastly, *E. coli* 3950 pCT6 killed *C. elegans* at the same rate as 3950 pCT (Figure 5.12B). However, *S. Typhimurium* SL1344 rif^R pCT6 killed *C. elegans* at a significantly slower rate than *S. Typhimurium* SL1344 rif^R pCT (Chi squared test, $P = 0.0001$) (Figure 5.12A) and was impaired in its ability to adhere to and invade INT-407 cells (Figure 5.11, Appendix 20) (Adhesion, $P = 0.0003$, Invasion, $P = <0.0001$).

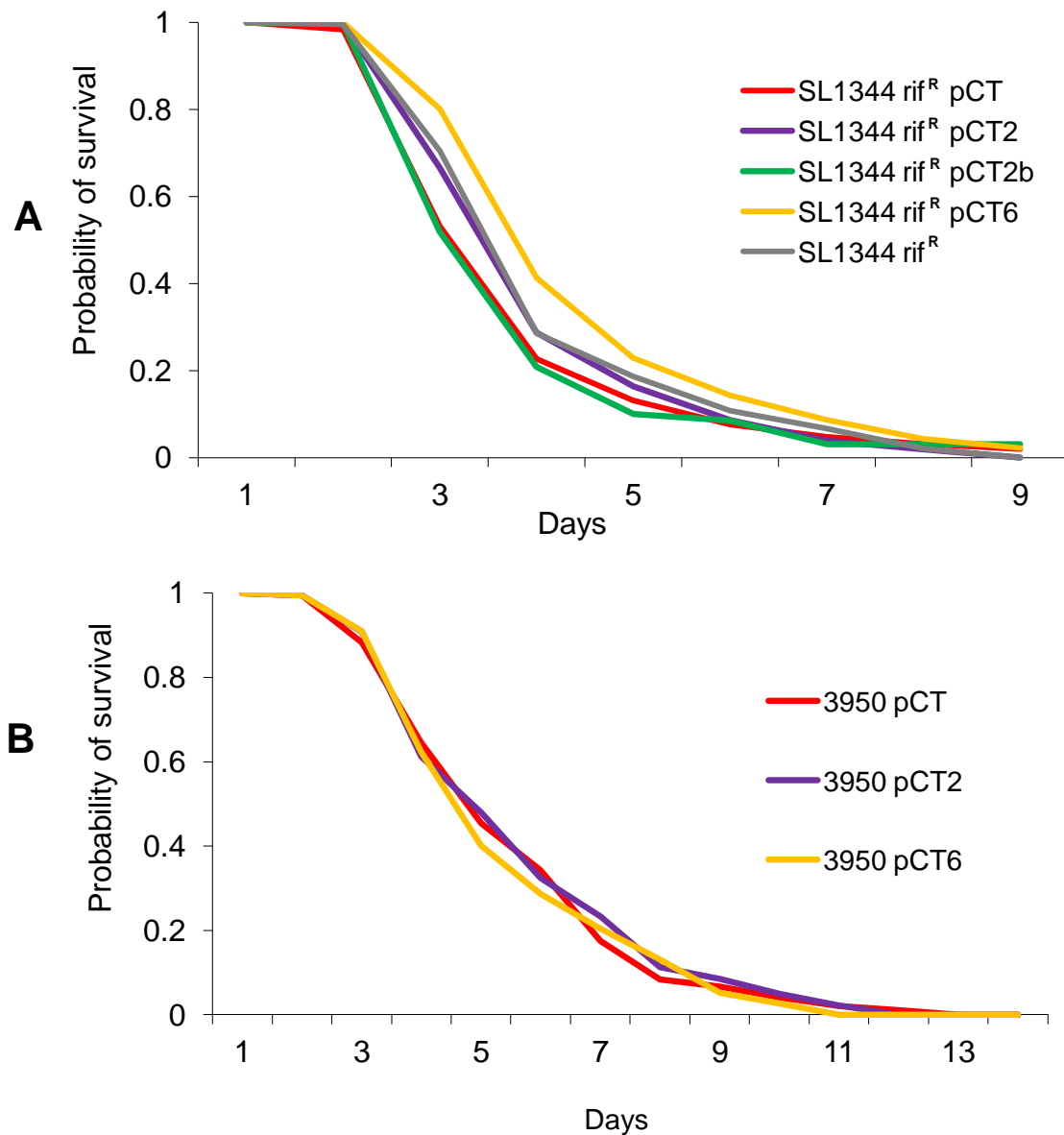
Figure 5.11. The ability of *S. Typhimurium* SL1344 rif^R with wild-type pCT and *bla*_{CTX-M-14} modified plasmids to adhere to and invade human intestinal cells



The number of *S. Typhimurium* SL1344 rif^R containing pCT2 (purple) pCT2b (green) or pCT6 (yellow) able to adhere to (A) and invade (B) INT-407 human intestinal cell is represented as a percentage of the number of *S. Typhimurium* SL1344 rif^R pCT (red) able to do so under test conditions (Appendix 20). Data are displayed as a mean of three separate experiments all performed with four technical repeats.

Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant are represented with an asterix.

Figure 5.12. The ability of bacterial hosts containing pCT2, pCT2b and pCT6 to cause infection the *C. elegans*



Survival curves were constructed using a Kaplan Mier calculation showing the probability of *C. elegans* survival each day when fed continuously on the tested bacterial strains.

A significance difference was found in the killing rate between *S. Typhimurium* SL1344 rif^R pCT and *S. Typhimurium* SL1344 rif^R pCT6 (*ISEcp1::aph*) ($P = 0.0001$). A significant difference in killing rate was defined as a P value <0.05 (Appendix 21).

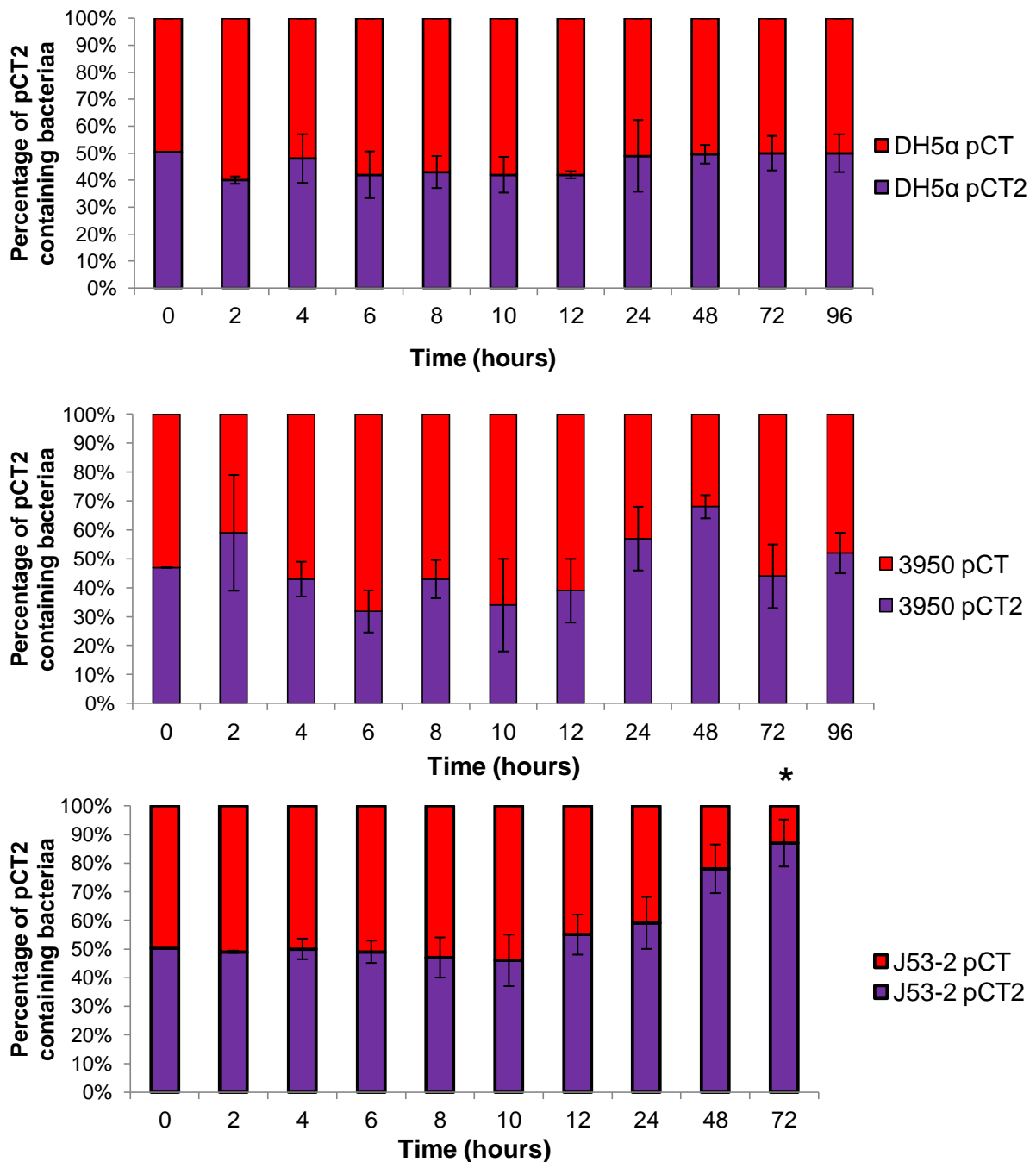
5.9 Pair-wise competition *in vitro* of hosts containing pCT and pCT2

To assess the competitive fitness of pCT +/- the *bla*_{CTX-M-14} gene in *E. coli* strains, pCT2 containing bacteria were competed in a 1:1 ratio against the corresponding host containing wild-type pCT *in vitro* on three separate occasions. During the first twelve hours, samples were collected every two hours and the proportion of bacteria carrying each plasmid calculated. After this time, samples were taken and the mixture was passaged into fresh LB broth every 24 hours for up to 96 hours, ~70 generations. In all three *E. coli* host strains (DH5 α , J53-2 and 3950) the plasmids remained in a 1:1 ratio during the first 12 hours, before the first passage (Figure 5.13, Appendix 22). Plasmids competed in *E. coli* DH5 α remained in a 1:1 ratio throughout the experiment and after four passages. In *E. coli* 3950 pCT and pCT2 also remained in a 1:1 ratio, however, there was more variation in the values obtained. In *E. coli* J53-2 the proportions of bacteria containing pCT2 increased during the experiment such that after 72 hours (three passages) pCT2 was found in 87% (Competition Index = 1.071).

5.10 *In vitro* competition between *E. coli* J53-2 and *E. coli* J53-2 pCT2

In chapter 4, wild-type pCT was shown to confer a fitness cost on *E. coli* host strain J53-2. In Section 5.9 of this chapter, *E. coli* J53-2 pCT2 was shown to have a competitive advantage over *E. coli* J53-2 pCT suggesting that pCT2 may confer less of a fitness cost to this host than pCT. Therefore, *E. coli* J53-2 pCT2 was competed in a 1:1 ratio with parental strain *E. coli* J53-2 to assess the fitness burden conferred by pCT2 (*bla*_{CTX-M-14}::*aph*) on *E. coli* J53-2 (Figure 5.14).

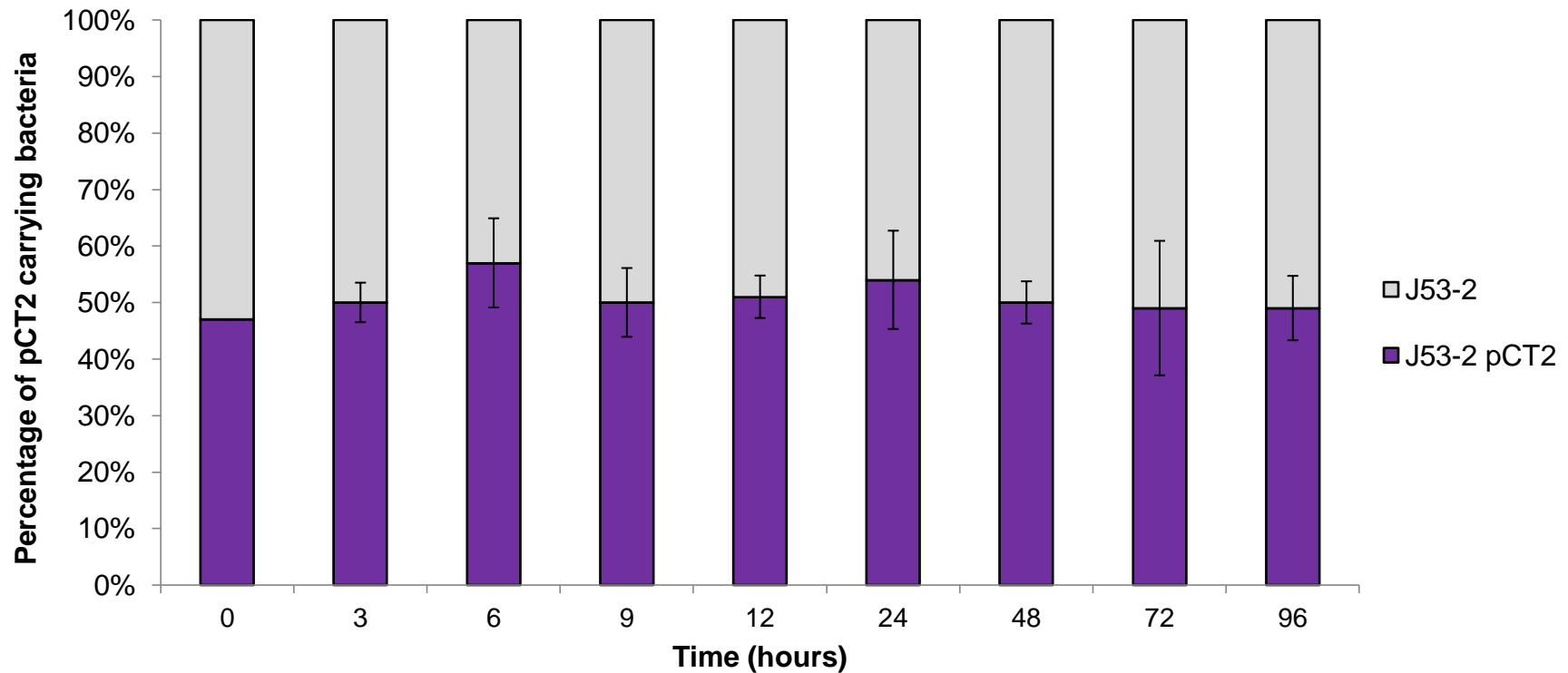
Figure 5.13. Competition between *E. coli* hosts carrying pCT and pCT2



The percentage of plasmid pCT2 carrying bacteria was calculated over time when *E. coli* strains carrying pCT or pCT2 were co-cultured in a 1:1 ratio *in vitro* (Appendix 22). A, The proportion of pCT2 carrying bacteria in a DH5α pCT:pCT2 co-culture; B, The proportion of pCT carrying bacteria in a 3950 pCT:pCT2 co-culture; C, The proportion of pCT carrying bacteria in a J53-2 pCT:pCT2 co-culture. Cultures were passaged into fresh broth at hour 12, 24, 48, 72 and 96 hours.

CI, competition index of the pCT containing strain in each pairing. An asterisk represent a statistically significant change in the proportion of bacteria containing pCT ($P < 0.005$).

Figure 5.14. Pair-wise competitive growth *in vitro* between *E. coli* J53-2 and *E. coli* J53-2 pCT2 (*bla*_{CTX-M-14}::*aph*)



Competition index of *E. coli* J53-2 pCT2 at 12 hours (before first passage) = 1.0037, $P = 0.414$ (no significant change)

Competition index of *E. coli* J53-2 pCT2 at 96 hours (after three passages) = 0.9996, $P = 0.286$ (no significant change)

The percentage of *E. coli* J53-2 bacteria carrying pCT2 over time was calculated when *E. coli* J53-2 +/- pCT2 was co-cultured in a 1:1 ratio *in vitro*. Cultures were passaged at hour 12, 24, 48 and 72 hours. The competition index was calculated from averages generated from at least three separate experiments.

The proportion of bacteria containing pCT2 remained at a 1:1 ratio throughout the duration of the experiment (CI = 0.9996 at 96 hours). This data suggests that the inactivation of the pCT *bla*_{CTX-M-14} gene (or the presence of the *aph* gene) decreases the pCT fitness burden on host strain *E. coli* J53-2, however did not alleviate the cost to allow spread of pCT throughout the population, as seen in host strains *E. coli* DH5α or *E. coli* 3950 (Figure 4.2).

5.11 Discussion

The relative advantages and disadvantages of a resident antibiotic resistance gene on the ability of large natural plasmids such as pCT to persist and spread in the absence of selective pressure is largely unknown. In order to assess the role of the sole pCT resistance gene, *bla*_{CTX-M-14}, in the evolutionary fitness of the plasmid in the absence of antibiotic pressure, this gene was inactivated and the biological effects on plasmid and bacterial host were investigated.

To inactivate *bla*_{CTX-M-14} on pCT, a novel protocol was designed which allowed easy and rapid targeted inactivation using a chromosomally based λ -red recombinase. This method was also shown to be effective in inactivating *ISEcp1* and five additional pCT genes (Chapter 6). Although optimised to inactivate pCT genes, this straightforward protocol could be applied to the inactivation of genes found on any plasmid which can be successfully transformed and extracted from *E. coli* SW102. A problem encountered post-recombination was the presence of both wild-type and mutant plasmid within the same bacterium, either co-existing or as a monomer. Wild-type pCT was successfully removed from bacteria by cycling cells in the presence of kanamycin. It is possible that exposure to the Gam component of the λ -red recombinase (which inhibits host nucleases) caused problems in plasmid replication

which may have led to monomer formation (Silberstein and Cohen, 1987; Katashkina *et al.*, 2009). During repeated use of this method this problem was not encountered again, suggesting that it was not a common event (Chapter 6).

The biological effects of inactivating *bla*_{CTX-M-14} and *ISEcp1* on pCT plasmid stability and conjugative transfer were assessed. *bla*_{CTX-M-14} and *ISEcp1* did not contribute to the inherent ability of pCT to be maintained within a bacterial population as pCT2, pCT2b and pCT6 remained stable over ~70 generations in the absence of antibiotic pressure. These data suggest that pCT stability is determined by other plasmid encoded factors for example, possible functional partitioning or addiction systems (Hayes, 2003). The inactivation of *bla*_{CTX-M-14} and *ISEcp1* on pCT also had no statistically significant effect on pCT's capacity to transfer to plasmid free bacterial strains. Of interest, pCT2 and pCT6 consistently conjugate at a higher rate than pCT, and their transfer frequencies were very similar under many of the conditions tested. As both plasmids gained additional DNA during the recombination process (pCT2 was 888 bp and pCT6 was 484 bp larger than wild-type pCT after recombination) it may have been predicted that these plasmids would take longer to transfer than pCT due to a longer replication time, although this effect may have been modest. The simplest explanation for increased frequency of conjugation is that the addition of the kanamycin resistance gene *aph*, which is common to both plasmids, had a positive effect on the conjugation process perhaps affecting a repressive regulatory mechanism (Haft *et al.*, 2006). Alternatively, it is tempting to speculate that the inactivation of *bla*_{CTX-M-14} (pCT2) and removal of a possible promoter (pCT6) may have alleviated a metabolic cost on the donor cell. This may allow a more rapid frequency of transfer (or altered transfer regulation).

pCT2b had a decreased conjugation frequency when compared to pCT. As pCT2b is 1485 bp larger than wild-type pCT (due to the insertion of *aph-gfpmut2*) and encodes a constitutively expressed stable GFP, it may be that pCT2b confers a larger cost on the host bacterial cell. Wendland and Bumann (2005) showed that the production of GFP conferred a metabolic burden and became toxic to host cells when expressed at high levels. As the copy number of pCT and pCT2b has not been determined it is unclear how many copies of *gfpmut2* were present in each cell and therefore whether production of GFP is likely to have reached toxic levels. However, there is little evidence in the literature that production of GFP has a negative effect on conjugation rates, and published studies have marked plasmids with *gfp* in order to measure plasmid transfer (Dahlberg *et al.*, 1998; Sorensen *et al.*, 2005). In my study, there was a reduction in the rate of pCT2b transfer in particular to recipient *E. coli* J53-2, suggesting host specific factors either related to the typically high transfer rate into this recipient or perhaps greater sensitivity to GFP.

The effects of inactivating *bla*_{CTX-M-14} on pCT host phenotypes were assessed by measuring pathogenicity in two infection model systems, growth kinetics and competitive fitness. Bacterial host strains containing pCT2 adhered to, and invaded tissue culture cells and killed *C. elegans* at an equivalent rate to strains containing pCT. These data suggest that inactivation of the *bla*_{CTX-M-14} gene had no effect on the ability of the bacterial host to cause infection when compared to hosts containing pCT. This corresponds with the findings of Dubois *et al.* (2009) who showed that the addition of *bla*_{CTX-M-1} to *E. coli* DH5 α did not confer a change in virulence when measured in human brain micro-vascular endothelial cells and in a mouse meningitis model. *S. Typhimurium* SL1344 *rif*^R pCT2b invaded human intestinal cells at a

significantly lower rate than SL1344 *rif^R* pCT. The less invasive nature of GFP producing bacteria is well documented in the literature and is suggested to be a result of a reduction in the ability of the GFP producing bacterium to adapt to the stress of intracellular life due to the extra stress already imposed by GFP (Knodler *et al.*, 2005).

The effect of inactivating *bla_{CTX-M-14}* on pCT host growth in the absence of cefotaxime was initially assessed by comparing the growth rates and generation times during the mid-logarithmic phase of each host strain harbouring either pCT or pCT2. No significant difference was found in any of the four bacterial host strains investigated. Recombination of *bla_{CTX-M-14}* with *aph-gfpmut2* also had no effect on host growth, except in host *E. coli* DH5 α where the addition of pCT2b resulted in a decreased generation time, compared to *E. coli* DH5 α pCT. However, it should be noted that this finding was not replicated when growing DH5 α pCT2b continuously shaking in larger volumes. It is important to note that the generation times calculated from growth in the FLUOstar OPTIMA do not necessarily reflect the generation times in other growth conditions due to reduced aeration, shaking and smaller culture volumes, therefore generation values are only comparable and relative to one another.

My data indicates that *in vitro* growth of host bacteria containing pCT was unaffected by the presence or absence of *bla_{CTX-M-14}*, suggesting this gene confers neither a fitness cost nor benefit to the host bacterium in the absence of antibiotic selective pressure. This was further supported by the finding that pCT and pCT2 remained in a 1:1 ratio when cultured together in host strains *E. coli* DH5 α and *E. coli* 3950, confirming that the inactivation of *bla_{CTX-M-14}* had no affect on the plasmids competitive fitness in these strains in the absence of antibiotic selective pressure.

However, in the third host *E. coli* J53-2, pCT2 had a significant fitness benefit over *E. coli* J53-2 pCT. pCT2 was also shown to confer less of a fitness cost upon host strain *E. coli* J53-2 than pCT, suggesting that the inactivation of *bla*_{CTX-M-14} (or/and addition of the *aph* gene) reduces the burden of the plasmid on this host strain.

There are many examples of β -lactamases which confer a metabolic cost upon their host bacteria. Those β -lactamase genes under inducible promoters, for example *bla*_{AMP-C}, are often found to have less of a fitness cost than when the same genes were constitutively expressed (Morosini *et al.*, 2000; Moya *et al.*, 2008). Similarly, *bla*_{CMY-7} had a higher fitness cost when located on high copy number plasmids than on low copy number plasmids or on the chromosome (Hossain *et al.*, 2004). The copy numbers of pCT and pCT2 in the various host strains were not determined in this study. If differences exist in the pCT copy number when in different hosts, or between pCT and pCT2 in the same host, this will determine how many copies of the *bla*_{CTX-M-14} gene are present in one cell. If pCT has a higher copy number than pCT2 in *E. coli* J53-2 this would result in a greater metabolic burden and may explain the results obtained. Mroczkowska and Barlow (2008) showed an enzyme specific effect when bacteria encoding *bla*_{TEM-1} outcompeted the same host carrying *bla*_{TEM-10} and *bla*_{TEM-12}. Similarly, *bla*_{SME-1} and *bla*_{AMP-C} conferred a greater fitness burden in certain bacterial host strains (*E. coli* K12 and *S. Typhimurium* SL1344 respectively)(Morosini *et al.*, 2000; Marciano *et al.*, 2007).

Interaction between plasmid and chromosomal resistance genes has also been shown to have a fitness effect on the host bacteria. Silva *et al.* (2011) examined epistasis between plasmid and chromosomally encoded antibiotic resistance genes. In many cases the fitness burden when carrying both chromosomal and plasmid

antibiotic resistance genes did not amount to the sum of the costs of these antibiotic resistance determinants when found separately. In one case the presence of *gyrA* mutation D87G and carriage of IncB plasmid R16 (conferring antibiotic resistance to ampicillin, kanamycin, streptomycin and sulphonamides tetracycline) alleviated the burden imposed by either plasmid or chromosomal mutation alone (Silva *et al.*, 2011). These findings may have implications in this study as *E. coli* J53-2 also has an *rpoB* mutation, it may be that interaction between pCT and/or *bla*_{CTX-M-14} with resistance genes found on the *E. coli* J53-2 chromosome account for the differences seen when using this host strain.

Despite the high conjugation rate of pCT to *E. coli* J53-2 recipients observed in the conjugation assays, the data collected during the pair-wise competition assay may suggest that pCT and pCT2 do not conjugate to plasmid free bacteria under these conditions. This may explain why if pCT also confers a fitness cost upon host *E. coli* J53-2, this plasmid is rapidly lost from a mixed population when it appears stable when grown isogenically. It would also explain why *E. coli* J53-2 pCT2 remains in a 1:1 ratio with J53-2 instead of disseminating to plasmid free bacteria as seen with the other *E. coli* bacterial host strains. Alternatively the results from co-culture of *E. coli* J53-2 and *E. coli* J53-2 pCT2 may reflect a balance of the rate of plasmid transfer with the rate at which *E. coli* J53-2 pCT2 is outcompeted by *E. coli* J53-2.

A limitation to measuring relative fitness of strains carrying plasmids using a direct pair-wise competition *in vitro* is the potential transfer of plasmids within the assay, thereby preventing the accurate assessment of the proportion of cells for example *E. coli* DH5α pCT vs. *E. coli* DH5α pCT2. To resolve this issue the proportion of host strains carrying either pCT or pCT2 at each time point was measured rather than the

hosts themselves. A more complex study design to examine competitive fitness could have marked host strains either within the chromosomal DNA (Grant *et al.*, 2008) or using fluorescence markers (e.g. *gfp*, *rfp*). This strategy may have elucidated the level and nature of transfer within each pair-wise competition experiment; however ensuring each combination of variables (e.g. marker, plasmid and resistance gene) did not have a different fitness cost on the host strain would have been very difficult. Therefore the simpler experimental design was used and deemed sufficient to answer whether pCT2 was more or less evolutionary fit than wild-type pCT.

Another limitation of this study was that *bla*_{CTX-M-14} was inactivated by replacement with an alternative resistance gene (*aph*) rather than complete removal of *bla*_{CTX-M-14}. This strategy was used due to the technical difficulties associated with genetic manipulation without a selective marker, but also so that kanamycin could be used to ensure maintenance of the plasmid and selection between hosts harbouring pCT or pCT2 during the competition experiments. Similarly, the marking of pCT by the addition of *gfp* was controlled for by recombining this gene into *bla*_{CTX-M-14} so any *gfp* specific effects would be highlighted when comparing pCT2b to pCT2.

When insertion sequence *ISEcp1*, was placed upstream of *bla*_{CTX-M-19} (a single point mutation variant of *bla*_{CTX-M-14}) the expression of this antibiotic resistance gene was up-regulated (Poirel *et al.*, 2003). It has been proposed that *ISEcp1* also up-regulates *bla*_{CTX-M-14} when located upstream of this gene on plasmids. To investigate whether *ISEcp1* has an influence on pCT fitness or the susceptibility of host strains to β -lactams, 1,008 bp of this element was removed and replaced with *aph* (pCT6). Host strains containing this plasmid had a comparable susceptibility to β -lactam antibiotics as hosts harbouring wild-type pCT, indicating that *ISEcp1* has no significant effect on

the β -lactamase gene. *S. Typhimurium* SL1344 rif^R pCT6 had altered growth kinetics and was also attenuated in both the *C. elegans* killing model and in its ability to adhere to and invade tissue culture cells when compared to *S. Typhimurium* SL1344 rif^R pCT. An explanation for this effect is difficult as the same effect was not observed with pCT2, therefore it is unlikely to be due to a down regulation of β -lactamase gene expression. It is possible that the *ISEcp1* transposase can interact with the *S. Typhimurium* SL1344 rif^R chromosome.

Analysis of the pCT sequence showed the presence of three putative promoter regions encoded within the pCT *ISEcp1* (Figure 5.15), the first of which shows the most homology with the proposed *ISEcp1* promoter of *bla*_{CTX-M-19} (Poirel *et al.*, 2003; Poirel *et al.*, 2005). Therefore inactivation of *ISEcp1* focused on these putative promoter sequences. Further investigation of this region revealed a fourth putative promoter sequence found 116 bp upstream of *bla*_{CTX-M-14} (-35 TTGAAA and -10 TGTTACAAT) previously not described in other *bla*_{CTX-M} carrying plasmids (as of September 2011). While the 42 bp found directly upstream of the pCT *bla*_{CTX-M-14} are homologous to the upstream region of almost all described *bla*_{CTX-M-14} genes (Kim *et al.*, 2011), the further 207 bp found within the pCT spacer region are unusual. This fourth promoter region is predicted to be a strong promoter of *bla*_{CTX-M-14} due to its proximity to the start codon and the high level of homology to the consensus promoter sequence. It is hypothesised that this fourth promoter is primarily responsible for the expression of *bla*_{CTX-M-14}. If this is the case then promoter sequences within *ISEcp1* become less important for pCT than in other published plasmids, and possibly accounts for an absence of a biological effect on inactivating the pCT *ISEcp1*.

Figure 5.15. Sequence of the pCT *ISEcp1* element and downstream spacer region marked with putative promoters and the site for recombination

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ATGATTAATAAAATTGATTTCAAAGCTAAGAATCTAACATCAAATGCAGGTCTTT
TTCTGCTCCTTGAGAATGCAAAAAGCAATGGGATTTTTGATTTTATTGAAAATGA
CCTCGTATTTTGATAATGACTCAACAAATAAAATCAAGATGAATCATATAAAGACC
ATGCTCTGCGGTCACTTCATTGGCATTGATAAGTTAGAACGTCTAAAGCTACTTC
AAAATGATCCCCTCGTCAACGAGTTTGATATTTCCGTAAAAGAACCTGAAACAGT
GTCACGGTTTTCTAGGAACTTCAACTTCAAGACAACCCAAATGTTTAGAGACATT
AATTTTAAAGTCTTTAAAAAACTGCTCACTAAAAGTAAATTGACATCCATTACGA
TTGATATTGATAGTAGTGTAATTAACGTAGAAGGTCATCAAGAAGGTGCGTCAAA
AGGATATAATCCTAAGAACTGGGAAACCGATGCTACAATATCCAATTTGCATTT
TGCGACGAATTAAAGCATATGTTACCGGATTTGTAAGAAGTGGCAATACTTACA
CTGCAAACGGTGCTGCGGAAATGATCAAAGAAATTGTTGCTAACATCAAATCAGA
CGATTTAGAAATTTTATTTTTCGAATGGATAGTGGCTACTTTGATGAAAAAATTATC
GAAAAGATAGAATCTCTTGGATGCAAATATTTAATTAAAGCCAAAAGTTATTCTA
CACTCACCTCACAAGCAACGAATTCATCAATTGTATTCGTTAAAGGAGAAGAAGG
TAGAGAACTACAGAACTGTATACAAAATTAGTTAAATGGGAAAAAGACAGAAGA
TTTGTCTGATCTCGCGTACTGAAACCAGAAAAAGAAAGAGCACAAATTATCACTTT
TAGAAGGTTCCGAATACGACTACTTTTTCTTTGTAACAAATACTACCTTGCTTTC
TGAAAAAGTAGTTATATACTATGAAAAGCGTGGTAATGCTGAAAACATATACAA
GAAGCCAAATACGACATGGCGGTGGGTCATCTCTTGCTAAAGTCATTTTGGGCGA
ATGAAGCCGTGTTTCAAATGATGATGCTTTCATATAACCTATTTTTGTTGTTCAA
GTTTGATTCCTTGGACTCTTCAGAATACAGACAGCAAATAAAGACCTTTCGTTTG
AAGTATGTATTTCTTGCAGCAAAAATTAATCAAACCGCAAGATATGTAATCATGA
AGTTGTCGGAAAACTATCCGTACAAGGGAGTGTATGAAAAATGTCTGGTATAATA
AGAATATCATCAATAAAATTGAGTGTTGCTCTGTGGATAACTTGCAGAGTTTATT
AAGTATCATTGCAGCAAAGATGAAATCAATGATTTATCAAAAATGATTGAAAGGT
GGTTGTAAATAATCTTACAATGTGTGAGAAGCAGTCTAAATTCTTCGTGAAATAG
TGATTTTTGAAGCTAATAAAAAACACACGTGGAATTTAGGGAATACTGATGTAAC
ACGGATTGACCGTATTGGGAGTTTGAGATGGTGACAAAGAGAGTGCAACGGATGA

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Black text, *ISEcp1* sequence remaining after recombination with *aph* PCR construct,
White text, DNA removed by recombination with the *aph* PCR construct,
Pink highlighted text, predicted promoter regions (-35 and -10),
Yellow highlighted text, binding regions of recombination primers,
Grey text, 249 bp spacer region between *ISEcp1* and *bla*_{CTX-M-14},
Blue text, *bla*_{CTX-M-14}.

In conclusion, inactivation of ESBL gene *bla*_{CTX-M-14} on plasmid pCT restored host susceptibility to β -lactam antibiotics but had no significant effect on the stability and transfer frequency of the plasmid, on growth rates of host bacterial strains or the ability of host bacteria to cause infection. Inactivation of *bla*_{CTX-M-14} conferred neither a fitness cost or fitness benefit when pCT2 was directly competed with the wild-type pCT *in vitro* in two out of three *E. coli* hosts. Replacement of the *ISEcp1* element and associated promoter sequences with *aph* had no effect on host susceptibility to cefotaxime and had no biological effect on *E. coli* DH5 α , J53-2 and 3950 host strains. These data indicate that this resistance gene has little effect on plasmid and host biological functions, and in the absence of antibiotic pressure is unlikely to have made a significant contribution to the success of pCT. Therefore, as *bla*_{CTX-M-14} is the only antibiotic resistance gene encoded on pCT, other plasmid backbone factors will determine persistence, dissemination and success of pCT in the absence of antibiotic pressure.

5.12 Further work

Several questions were raised by the inactivation of *bla*_{CTX-M-14} and upstream insertion sequence *ISEcp1*. To further investigate interactions between hosts, plasmids and *bla*_{CTX-M-14}, this gene could be cloned or recombined into other plasmid backbones, either a well characterised molecular vector such as pBR322 (as in the early fitness experiments)(Lee and Edlin, 1985); or onto another large natural plasmid which lacks a β -lactamase gene. The fitness effects of *bla*_{CTX-M-14} on a naive plasmid and other bacterial host strains could then be assessed to suggest whether pCT may have adapted to accommodate the resistance gene after acquisition, or whether *bla*_{CTX-M-14} inherently confers no or little cost in the absence of selective

pressure. The integration of other clinically relevant resistance genes such as *bla*_{NDM-1}, *bla*_{CTX-M-15} or *bla*_{CMY-2} into the pCT genome would also allow the investigation of whether pCT has co-evolved to tolerate *bla*_{CTX-M-14} specifically or whether the backbone is unperturbed by the addition of potentially costly resistance genes.

If this study were to be repeated with the knowledge that the plasmid remains stable in the absence of selective pressure over at least 70 generations, complete removal of *bla*_{CTX-M-14} may have been a better study design. After recombination with *aph*, pCP20 as is described by Datsenko and Wanner (2000) would be used to remove the *aph* cassette. While technically difficult this would allow a cleaner assessment of the role of *bla*_{CTX-M-14} in pCT success. Alternatively, inactivation of the *bla*_{CTX-M-14} gene could use a range of selective markers e.g. chloramphenicol resistance gene *cat* or tetracycline resistance gene *tetO* to further investigate the effects of inactivating *bla*_{CTX-M-14} versus the insertion of a new resistance marker.

Another key question remaining is whether *bla*_{CTX-M-14} is differentially expressed, either in different hosts, under different growth conditions, and when putative promoters within the *ISEcp1* element have been removed. To investigate this RNA should be extracted from each host strain containing pCT, pCT2 and pCT6 and the levels of *bla*_{CTX-M-14} mRNA compared using RT-PCR. There are inherent problems with this straightforward method. The first is that a change in plasmid copy number would increase the level of mRNA within a cell therefore confounding the data. To get around this, copy number in each of the conditions tested should also be measured using the expression levels of various genes around the plasmid genome (as discussed in chapter 4). One interesting aspect of an experiment of this kind would be investigation of the role the additional 'fourth' putative promoter found 116 bp

upstream of *bla*_{CTX-M-14} on expression of this resistance gene. Expression could be assessed by removing the promoter DNA sequence and using RT-PCR to measure expression changes; or by cloning this DNA region into an expression vector to first establish whether it is indeed functional. Investigation of whether the plasmid copy number changes when inactivating *bla*_{CTX-M-14} or *ISEcp1* (particularly in *E. coli* J53-2 and *S. Typhimurium* SL1344 *rif*^R respectively) would also be of value during future work.

In this study *bla*_{CTX-M-14} was found to remain stable on the pCT genome in all experiments conducted. However, strains were typically cultured for no more than 70-100 generations during persistence and fitness assays. In a recent study examining the fitness burden of *bla*_{CMY-2} carrying IncA/C plasmids, the loss of resistance genes was only seen after ~1000 generations (Subbiah *et al.*, 2011). Therefore if pCT carrying strains were cultured over this extended period in the absence of antibiotic it may be that resistance gene loss is also observed from pCT.

Due to the decreased pCT2b transfer rate in some of the conditions tested, any results obtained from this *gfp* marked pCT could not reliably be extrapolated to wild-type pCT. Marking pCT by insertion of *gfp* into an intergenic region (e.g. between pCT008 and pCT009) was proposed as a method for measuring pCT transfer *in vivo*, e.g. within the *C. elegans* intestinal tract and pCT *in-situ* migration within a biofilm (Hausner and Wuertz, 1999; Sorensen *et al.*, 2005). A *gfp* marked pCT could also have been used in high throughput transfer experiments using flow cytometry to detect the number of transconjugants under a range of conditions (Arango Pinedo and Smets, 2005). However, the observed reduction in pCT2b transfer compared to pCT and pCT2 in some of the conditions tests suggested that any results obtained

using a *gfp* tagged plasmid may not be applicable to wild-type pCT, therefore this plasmid was not created for investigation, but provides an interesting avenue for further work.

It has been hypothesised that in some instances β -lactamase enzymes may have a role in cell wall metabolism such as cell wall recycling (Bishop and Weiner, 1992; Alonso *et al.*, 2001), which may have a positive effect on the fitness of the bacterium and select for the maintenance of these genes in the absence of selective pressure. For further work investigating the role of *bla*_{CTX-M-14} in plasmid success, the cell wall integrity of hosts +/- pCT could be measured to look for positive effects of CTX-M-14. Similarly *Kluyvera georgiana* β -lactamase genes *klug1* could be functionally inactivated to look for signs of interaction with the cell wall integrity in their postulated natural host. These experiments may give further clues as to the evolutionary path of *bla*_{CTX-M} genes and the possible benefits they may confer to their hosts.

5.13 Key findings

- A novel and rapid method to inactivate genes on large conjugative plasmids was developed using a chromosomally encoded λ -red recombinase.
- *bla*_{CTX-M-14} on pCT was inactivated by recombination with an *aph* construct.
- Plasmid pCT was also successfully marked with *gfp* within *bla*_{CTX-M-14}.
- Inactivation of *bla*_{CTX-M-14} on pCT
 - restored host susceptibility to β -lactam cefotaxime, confirming the role of this gene in the resistant phenotype,
 - did not impair pCTs ability to persist within 100% of cells within a culture,
 - did not significantly change the pCT transfer frequency,

- had no affect on the ability of host strains to cause infection,
 - had no significant effect on the growth rate of host strains grown isogenically,
 - did not confer a fitness cost or benefit when host strains *E. coli* DH5α and *E. coli* 3950 containing either pCT or pCT2 were competed in a 1:1 ratio.
- Therefore inactivation of *bla*_{CTX-M-14} does not appear to confer either a fitness cost or benefit on pCT plasmid function or on host fitness (with the exception of host *E. coli* J53-2)
 - *E. coli* J53-2 pCT2 was found to have a significant competitive advantage over *E. coli* J53-2 pCT when strains were placed in competition *in vitro*, and conferred less of a fitness burden on host strain *E. coli* J53-2 than pCT.
 - The addition of *gfp* to pCT was shown to reduce conjugation frequency, particularly to recipient *E. coli* J53-2, and to impair the ability of *S. Typhimurium* SL1344 rif^R to adhere to and invade tissue culture cells.
 - Insertion sequence *ISEcp1* was found to encode three putative promoters, two of which were removed by recombination with *aph*. Inactivation of *ISEcp1* had no effect on pCT stability, no significant effect on transfer rate despite a small consistent increased frequency into all recipient hosts, and had no effect on growth or the ability of *E. coli* 3950 to cause infection. Host growth was also unaffected in *E. coli* J53-2 and *E. coli* DH5α.
 - Inactivation of *ISEcp1* (pCT6) in host *S. Typhimurium* SL1344 rif^R showed attenuation in both infection models and a decreased growth rate when compared to *S. Typhimurium* SL1344 rif^R pCT.

Chapter 6:

The role of five pCT genes in the “success” of pCT

6. The role of five pCT genes in the “success” of pCT

6.1 Hypotheses and Aims

In previous chapters pCT was reported to have spread around the world and showed to confer no fitness cost or benefit upon host bacterial strains *E. coli* DH5 α and *E. coli* 3950. Inactivation of antibiotic resistance gene *bla*_{CTX-M-14} also had no effect on the ability of pCT to persist and disseminate in the absence of antibiotic pressure in these host strains. Therefore it was hypothesised that genes other than *bla*_{CTX-M-14} encoded within the pCT backbone have contributed to the ‘success’ of this plasmid.

The aims of this section were to:

- Select and inactivate five genes hypothesised to contribute to pCT success,
- Investigate the contribution of *pndACB* in pCT persistence,
- Investigate the role of the *pil* and *tra* locus in pCT conjugation,
- Investigate the biological effects of inactivating the putative sigma factor gene,
- To explore other aspects of fitness such as biofilm formation and aggregation.

6.2 Inactivation of five pCT ‘backbone’ genes

6.2.1 Selection of genomic regions for further investigation

Through the pCT sequence analysis, comparative genomics and review of the literature, outlined in Chapter 3, five pCT genomic regions emerged as possible contributors to the successful spread and persistence of pCT.

6.2.1.1 *Putative sigma factor*

The putative RNA polymerase sigma factor encoded by pCT_066 was unusual compared to other sigma factors of the same family (σ 70). The lack of available

literature on the role of plasmid encoded sigma factors, and the potential for this gene to regulate transcription of plasmid or host genes made pCT_066 a candidate for further investigation.

6.2.1.2 *The pil locus and pilS*

In chapter 4 the relevance of pCT conjugation in both liquid media and on a solid surface was demonstrated. Previous studies have shown that the *pil* locus of IncI type plasmids is required for conjugation in liquid (Bradley, 1984; Yoshida *et al.*, 1999). Production of a thin pilus has also been associated with increased adherence to eukaryotic cells, polystyrene surfaces, other bacterial cells and to biofilm formation (Dudley *et al.*, 2006). *pilS* (pCT_103) was selected for inactivation, as it encodes a pre-pilin subunit (22 kDa) which is processed into a 19 kDa major structural pilin component. Removal of this gene in other IncI plasmids (R64 and pSERB1) prevented successful assembly of the type IV pilus (Dudley *et al.*, 2006; Shimoda *et al.*, 2008) thereby allowing investigation of the role of this conjugation apparatus.

6.2.1.3 *Shufflon region and shufflon recombinase (rci)*

The pCT shufflon region was inferred to be active due to the multiple forms of the *pilV* region within the plasmid sequence assembly data. It is likely that the shufflon regions of five closely related plasmids to pCT (pO113, pO26_vir, pR3521 and pSERB1) are static due to a missing *rci* or insertions within this region, therefore making the active nature of this feature in pCT distinct from other closely related plasmids. Komano *et al.*, (2005) showed that the functioning shufflon region of plasmid R64 influenced the conjugation frequency of the plasmid in liquid, and determined recipient specificity. Inactivation of the pCT shufflon recombinase (*rci*) should result in the fixing of the *pilV* shufflon components into one particular

conformation, preventing switching between the different organisations. Therefore, the effects resulting from an inactive shufflon region on the transfer of pCT and host fitness examined.

6.2.1.4 *The tra locus and traY*

The *tra* locus is required for plasmid transfer in liquid and on solid surfaces (Kim *et al.*, 1993), therefore this region was disrupted to create a non-conjugative pCT mutant and to assess the contribution of the thick pilus to pCT dissemination and 'success'. *traY* was selected for inactivation as it encodes an essential integral membrane protein required for successful pilus formation (Komano *et al.*, 2000; Sampei *et al.*, 2010).

6.2.1.5 *Putative addiction system pndACB*

pCT was stable in the absence of antibiotic pressure in four different bacterial hosts, including *E. coli* J53-2 in which pCT had a fitness cost (Chapter 4). These data suggest that pCT encodes a mechanism for effective partitioning, or an addiction system which ensures effective vertical plasmid transfer to all daughter cells. The initial annotation of the pCT sequence did not elucidate any complete addiction or partitioning systems. However, re-analysis of the sequence highlighted pCT_065 (*pndA*) which was annotated as a possible stability toxin but which seemed to lack a regulator (*pndC*) or antisense antitoxin (*pndB*). Alignments of pCT with characterised *pndACB* operons in plasmids R64, R483 and pO113 revealed that pCT did encode a complete *pndACB* operon, which had been incorrectly annotated (Nielsen and Gerdes, 1995; Furuya and Komano, 1996). In R64 the inactivation of *pndACB* had an extreme detrimental effect on plasmid stability (Furuya and Komano, 1996), therefore *pndACB* was selected for inactivation to assess the role of this gene in pCT stability.

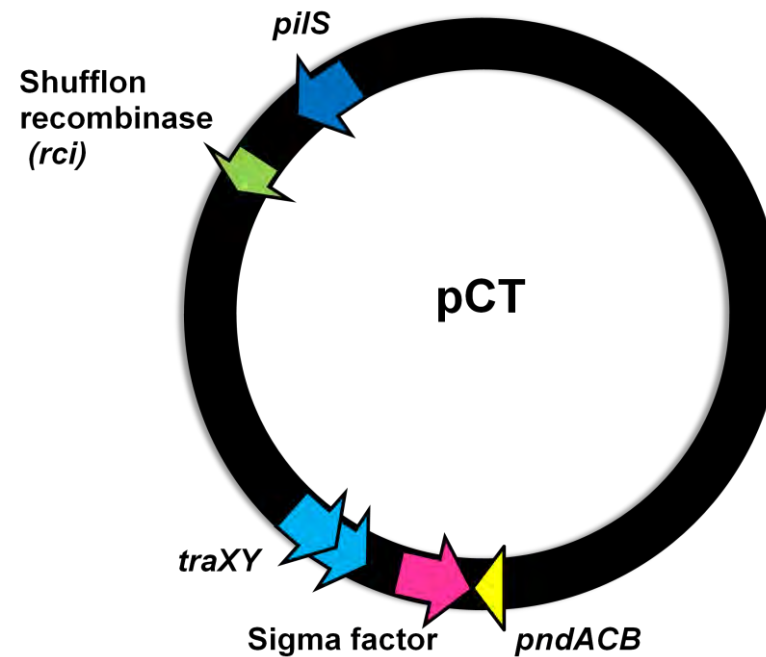
6.2.2 Inactivation of five pCT genes and verification

Inactivation of the five selected genes (pCT_066, *rci*, *pilS*, *traY* and *pndACB*) by recombination with *aph* was achieved using the method developed in Chapter 4 (Table 2.7; Appendix 23-28). In the case of *traY*, the upstream primer was designed with homology to adjacent CDS *traX* to obtain an optimum primer pair (Appendix 26). The DNA of each candidate mutant plasmid (each from one randomly selected colony) was extracted and transformed into *E. coli* DH5 α creating strains *E. coli* DH5 α pCT3 (Sigma factor::*aph*; I827), *E. coli* DH5 α pCT4 (*rci*::*aph*; I828), *E. coli* DH5 α pCT5 (*pilS*::*aph*; I829), *E. coli* DH5 α pCT7 (*traXY*::*aph*; I846) and *E. coli* DH5 α pCT8 (*pndACB*::*aph*; I877).

Recombination of *aph* into the pCT *pndACB* operon generated 380 colonies which were able to grow in the presence of kanamycin, indicating successful recombination. However, the majority of these colonies ceased to grow after approximately 12 hours (~1 mm in size), and when replica plated onto fresh agar plates (LB and LB containing 50 μ g/ml kanamycin) or cultured in LB broth, no growth was observed. Ten colonies out of the 380 continue to grow to >3 mm in size, therefore one of these colonies was selected and the plasmid DNA extracted.

Inactivation of each of the five genes was verified by PCR amplification across the genomic region and DNA sequencing (Appendix 23-28). The mutant plasmids (with the exception of pCT7 *traXY*::*aph*) were transferred by conjugation to *E. coli* host strains J53-2 (I847) and 3950 (I823), and to *S. Typhimurium* SL1344 *rif*^R (L1078)(Figure 6.1). pCT7 was unable to conjugate to recipients, and multiple attempts to transform this plasmid into all three alternative hosts were unsuccessful (Figure 6.1).

Figure 6.1. pCT genes selected for inactivation



| Plasmid | Inactivated gene | pCT ID | Resistance marker | Laboratory codes of plasmid containing host strains | | | |
|---------|-------------------------------------|-------------|---------------------------|---|-------|------|-------------------------|
| | | | | DH5α | J53-2 | 3950 | SL1344 rif ^R |
| pCT3 | Putative sigma factor | pCT_066 | <i>aph</i> | l827 | l858 | l837 | L1236 |
| pCT4 | Shufflon recombinase (<i>rci</i>) | pCT_093 | <i>aph</i> | l828 | l859 | l831 | L1259 |
| pCT5 | <i>pilS</i> | pCT_103 | <i>aph</i> | l829 | l860 | l841 | L1260 |
| pCT7 | <i>traXY</i> | pCT_069 | <i>aph</i> | l846 | - | - | - |
| pCT8 | <i>pndACB</i> | pCT_065 | <i>aph</i> | l877 | l893 | l894 | L1397 |
| pCT9 | <i>traXY</i> and <i>pndACB</i> | pCT_069/065 | <i>aph</i> and <i>cat</i> | l878 | - | - | - |

6.2.3 Creation of a mutant with both *traXY* and *pndACB* inactivated

In order to assess the effect of inactivation of *pndACB* on plasmid persistence and vertical transfer only, *pndACB* was also insertionally inactivated in non-transferrable pCT7 (in addition to pCT). pCT7 was transformed into the λ -red recombinase encoding *E. coli* strain SW102, to create strain *E. coli* SW102 pCT7 (I880). The same primers designed to inactivate *pndACB* with *aph* (Table 2.7, primers 1354/1355) were used to amplify the chloramphenicol resistance gene *cat* from the chromosome of *S. Typhimurium* L829 (L828 *tolC::cat*). The resulting PCR amplicon was recombined with the pCT7 *pndACB* as described previously (Section 6.2.2). Approximately 500 colonies grew on selective agar plates, however, as with the previous *pndACB* disruption (pCT8), only 18 colonies grew after 24 hours. The DNA was extracted from a randomly selected growing colony and transformed into *E. coli* DH5 α , creating strain *E. coli* DH5 α pCT9 (I878). DNA amplification and sequencing of *traXY* and *pndACB* verified the presence of *aph* and *cat* in appropriate regions.

6.3 Effects on pCT plasmid biology of inactivating the five genes

6.3.1 Stability of pCT3, pCT4, pCT5, pCT7, pCT8 and pCT9 in bacterial cells

The effects of inactivating the five selected genes on the ability of pCT to persist and to remain stable within a bacterial population without antibiotic selection was assessed *in vitro* as previously described (Section 4.4.1). The six plasmids (pCT3-5 and 7-9) were stably maintained in 100% of bacteria in the four host strains (*E. coli* DH5 α , *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R) over 96 hours (~70 generations). These data suggest that none of the selected pCT genes (including *pndACB*) are required for the successful short term persistence of pCT.

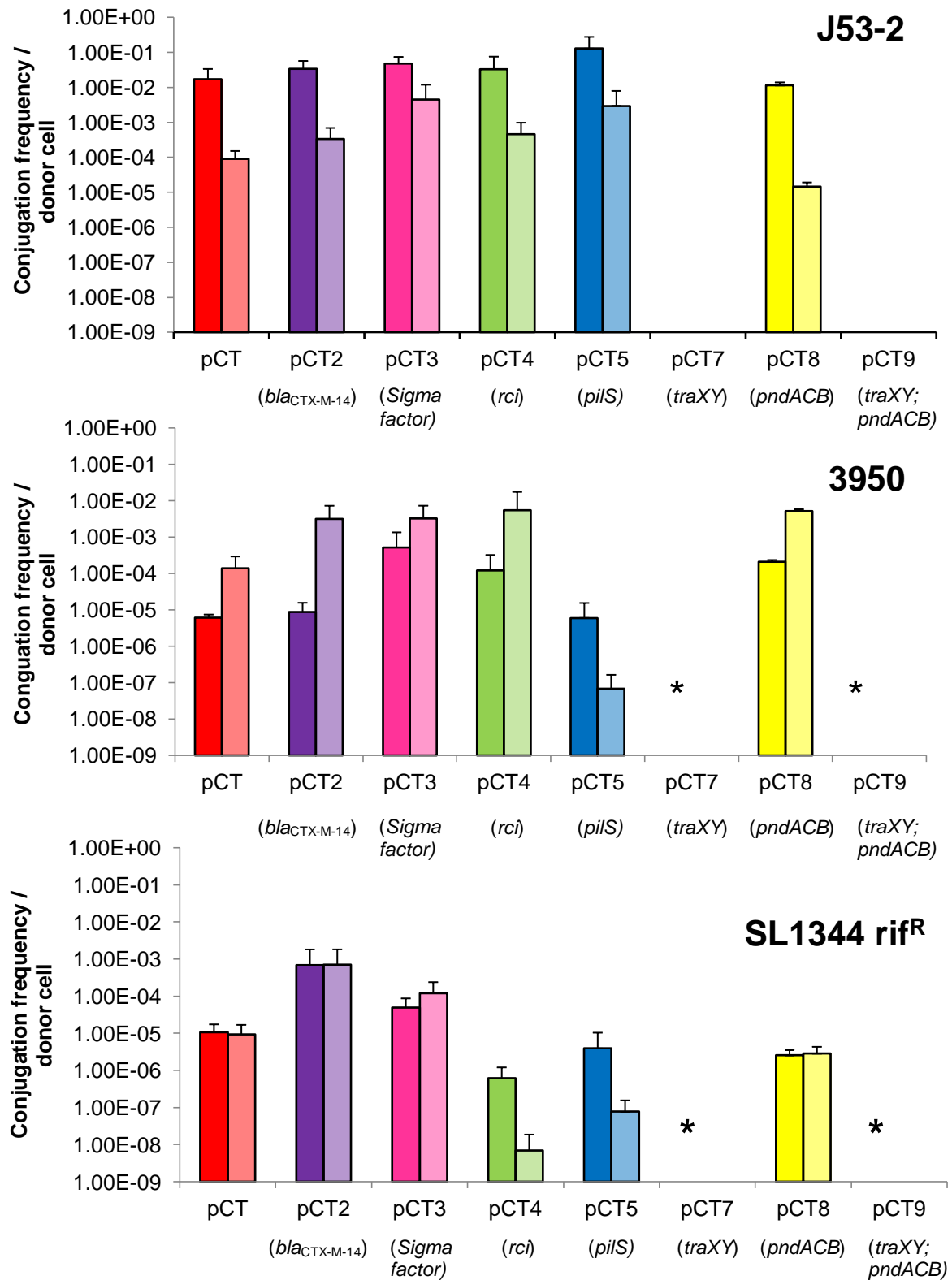
6.3.2 Conjugative transfer of pCT3, pCT4, pCT5, pCT7, pCT8 and pCT9

Plasmids pCT7 (*traXY::aph*) and pCT9 (*traXY::aph*, *pndACB::cat*), both lacking a functional *traY* were unable to transfer by conjugation to any of the recipient strains over 48 hours in broth or on a solid surface.

The conjugation frequencies of the other mutant plasmids (pCT3, pCT4, pCT5 and pCT8) were measured over a three hour period at 37°C from donor *E. coli* DH5α to recipients *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R, in liquid and on a solid surface on three separate occasions (Figure 6.2; Appendix 29). Transfer of pCT3-5 and pCT8 to recipient *E. coli* J53-2 was at a similar rate to that of pCT, suggesting that inactivation of the putative sigma factor, *pndACB* and *pil* locus related genes (*pilS* and *rci*) are not essential for pCT transfer to this recipient strain. Inactivation of *pilS* (pCT5) did not affect the frequency of transfer on a solid surface. However, the conjugation frequency of pCT5 (*pilS::aph*) to recipients *E. coli* 3950 (6.73×10^{-8}) and *S. Typhimurium* SL1344 rif^R (7.69×10^{-8}) in liquid was lower than of wild-type pCT (1.39×10^{-4} , 9.33×10^{-6} respectively).

Inactivation of the shufflon recombinase gene (pCT4) had no effect on conjugation to the *E. coli* recipient strains. However, there was consistently less transfer of pCT4 (*rci::aph*) on a filter and to a greater extent in liquid, to recipient *S. Typhimurium* SL1344 rif^R (Figure 6.2, Appendix 29). In every condition tested, the conjugation frequency of pCT3 (Sigma factor::*aph*) was consistently higher than that of pCT, however this difference was not found to be statistically significant.

Figure 6.2. Conjugation frequencies of plasmids from donor *E. coli* DH5 α to recipients *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R



Solid bars represent conjugation frequencies on filters and faded bars represent the conjugation frequency in liquid; an asterisk denotes a statistically significant difference compared to pCT. Gene names in brackets show the inactivated gene on each plasmid.

6.4 Effects of pCT3, pCT4, pCT5, pCT7, pCT8 and pCT9 upon bacterial host strains

6.4.1 Susceptibility of strains containing mutant plasmids to cefotaxime and ceftiofur

The susceptibilities of *E. coli* DH5 α , *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R containing the six mutant plasmids to cefotaxime and ceftiofur were compared to that of host strains containing wild-type pCT using the agar doubling dilution method. The MIC of the two β -lactams against strains containing plasmids pCT3, pCT5 and pCT7-9 were equivalent to that for pCT containing strains (Table 6.1). This indicates that the pCT sigma factor, *pil*, *tra* and *pndACB* loci have no effect on host susceptibility to β -lactams. Inactivation of the pCT shufflon recombinase (pCT4) resulted in a consistently increased MIC of cefotaxime by at least one doubling dilution in host strains *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R on five separate occasions (Table 6.1). However, as the error within this method is +/- one doubling dilution it is unclear whether this is a significant finding.

6.4.2 Growth kinetics of host strains containing pCT3-5 and pCT7-9

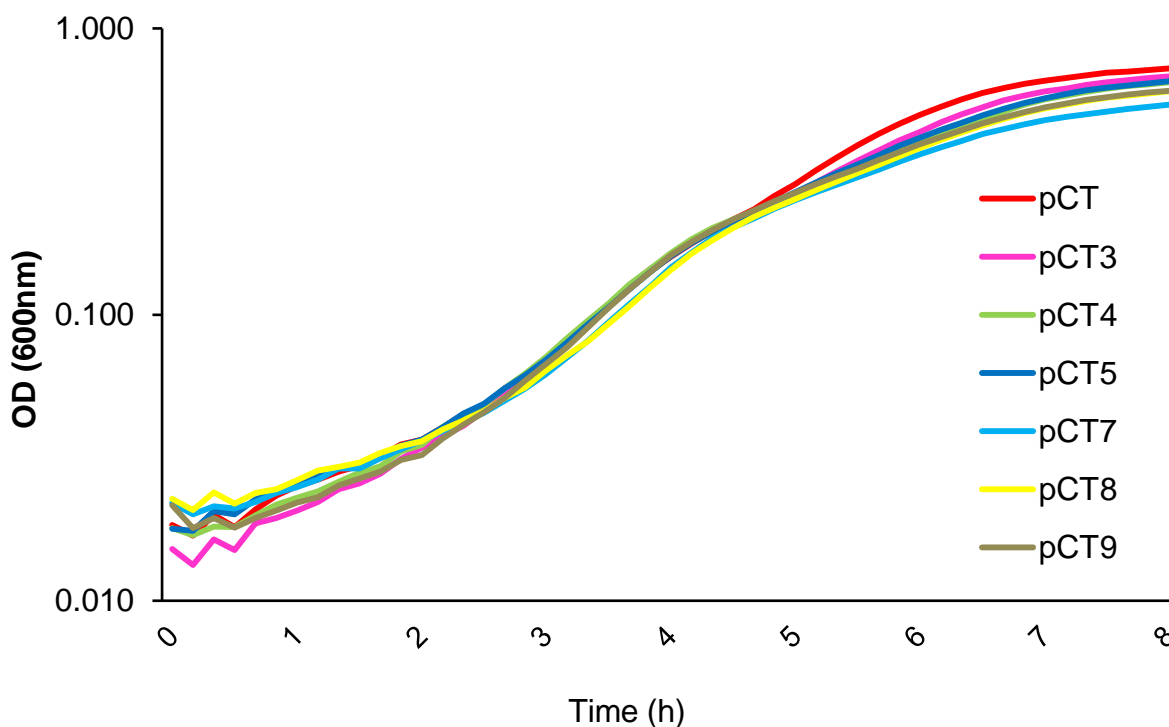
To assess whether inactivation of the five pCT genes had an effect on host growth *in vitro*, the growth kinetics and generation times of the host strains containing pCT and the six mutant plasmids were measured using a FLUOstar OPTIMA (Figure 6.3 and Figure 6.4). With the exception of *S. Typhimurium* SL1344 rif^R pCT3, no significant differences were found in the generation times of all hosts containing the six plasmids compared to hosts containing wild-type pCT. These data suggest that inactivation of the pCT sigma factor, *rci*, *pilS*, *traXY*, *pndACB* has no detrimental or beneficial effect on host growth in a pure culture.

Table 6.1. Minimum inhibitory concentrations of cefotaxime and ceftiofur for hosts containing pCT and the other modified plasmids

| Laboratory number | Strain | MIC of cefotaxime (µg/ml) | MIC of ceftiofur (µg/ml) |
|-------------------|--|---------------------------|--------------------------|
| I755 | DH5α pCT | 16 | 32 |
| I827 | DH5α pCT3 (Sigma factor:: <i>aph</i>) | 16 | 32 |
| I828 | DH5α pCT4 (<i>rci</i> :: <i>aph</i>) | 16 | 32 |
| I829 | DH5α pCT5 (<i>pilS</i> :: <i>aph</i>) | 16 | 32 |
| I846 | DH5α pCT7 (<i>traXY</i> :: <i>aph</i>) | 16 | 32 |
| I877 | DH5α pCT8 (<i>pndACB</i> :: <i>aph</i>) | 16 | 32 |
| I878 | DH5α pCT9 (<i>traXY</i> :: <i>aph</i> ; <i>pndACB</i> :: <i>cat</i>) | 16 | 32 |
| I834 | J53-2 pCT | 16 | 32 |
| I858 | J53-2 pCT3 (Sigma factor:: <i>aph</i>) | 16 | 64 |
| I859 | J53-2 pCT4 (<i>rci</i> :: <i>aph</i>) | 32 | 64 |
| I860 | J53-2 pCT5 (<i>pilS</i> :: <i>aph</i>) | 16 | 64 |
| I893 | J53-2 pCT8 (<i>pndACB</i> :: <i>aph</i>) | 16 | 64 |
| I855 | 3950 pCT | 16 | 64 |
| I837 | 3950 pCT3 (Sigma factor:: <i>aph</i>) | 16 | 64 |
| I831 | 3950 pCT4 (<i>rci</i> :: <i>aph</i>) | 32 | 64 |
| I841 | 3950 pCT5 (<i>pilS</i> :: <i>aph</i>) | 16 | 64 |
| I894 | 3950 pCT8 (<i>pndACB</i> :: <i>aph</i>) | 16 | 64 |
| L1079 | SL1344 rif ^R pCT | 64 | 64 |
| L1236 | SL1344 rif ^R pCT3 (Sigma factor:: <i>aph</i>) | 32 | 64 |
| L1259 | SL1344 rif ^R pCT4 (<i>rci</i> :: <i>aph</i>) | 256 | 128 |
| L1260 | SL1344 rif ^R pCT5 (<i>pilS</i> :: <i>aph</i>) | 32 | 64 |
| L1397 | SL1344 rif ^R pCT8 (<i>pndACB</i> :: <i>aph</i>) | 32 | 128 |

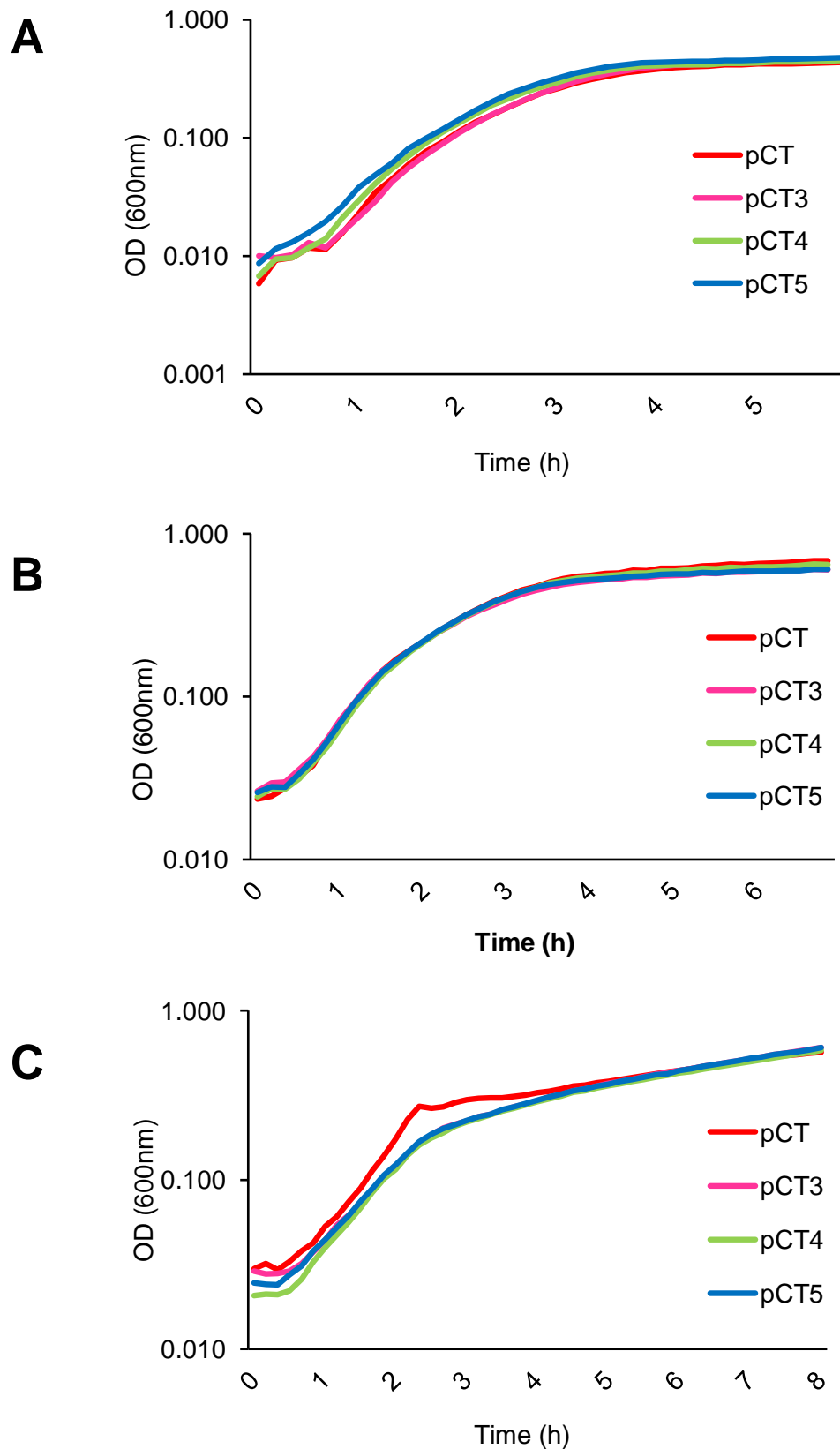
The MIC of each antibiotic is the mode of values obtained on at least three separate occasions, bold text denotes an altered MIC.

Figure 6.3. Growth kinetics of *E. coli* DH5 α containing pCT, pCT3-9



The growth kinetics of bacterial host strain *E. coli* DH5 α containing either pCT, pCT3 (Sigma factor::*aph*), pCT4 (*rci*::*aph*), pCT5 (*pilS*::*aph*), pCT7 (*traXY*::*aph*), pCT8 (*pndACB*::*aph*) or pCT9 (*traXY*::*aph*; *pndACB*::*cat*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 2-3 (Appendix 30). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted by an asterix.

Figure 6.4. Growth kinetics of host strains containing pCT, pCT3-5

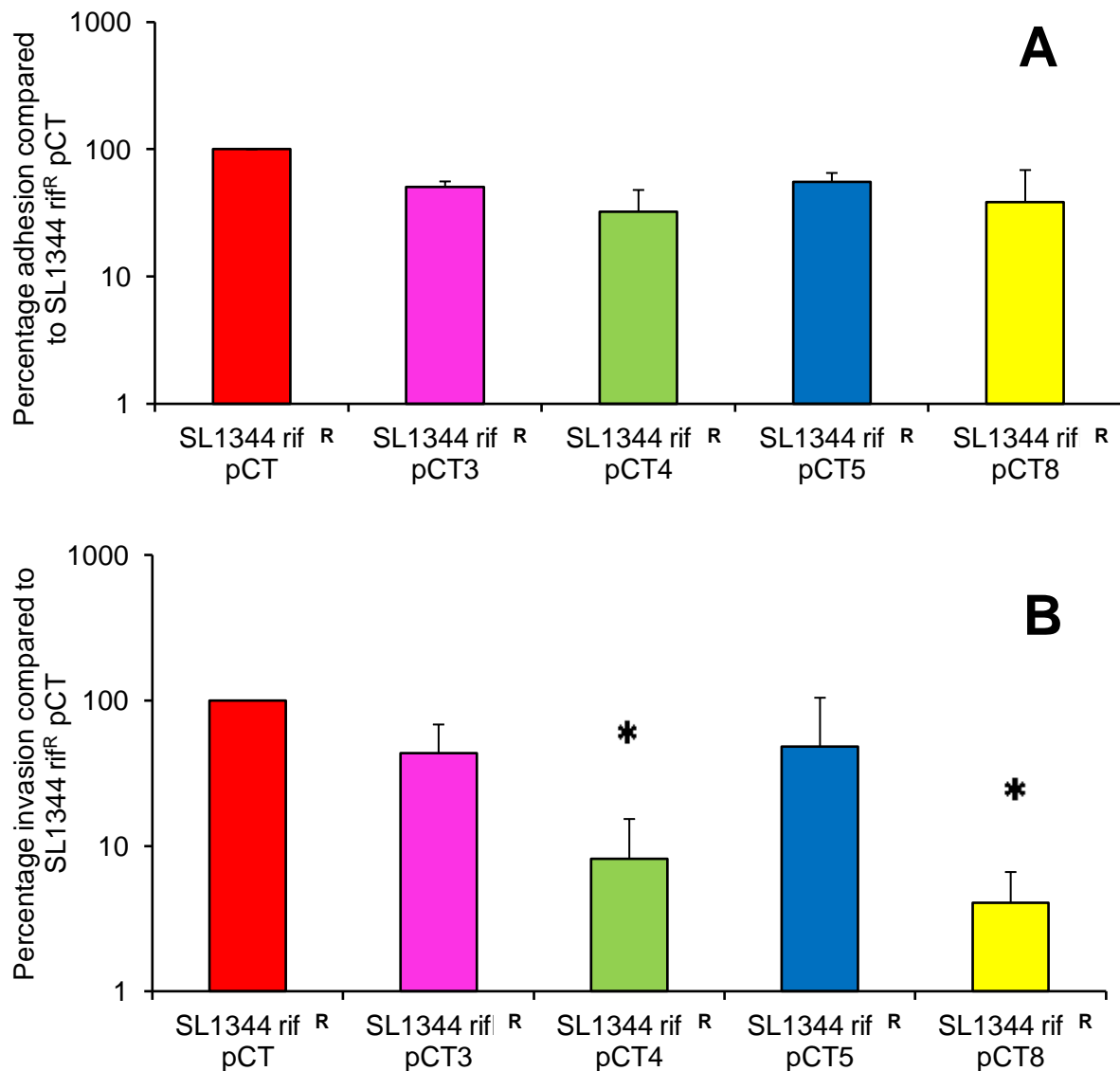


The growth kinetics of bacterial host strains containing either pCT, pCT3 (Sigma factor::*aph*), pCT4 (*rci::aph*) or pCT5 (*pilS::aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Appendix 31).

6.4.3 The effect of pCT3, pCT4, pCT5 and pCT8 on the ability of bacterial host strains to cause infection

To investigate whether the pCT putative sigma factor, *pil* locus, functional shufflon and the *pndACB* genes influence the ability of host bacteria to cause infection, the ability of bacterial hosts containing pCT3, 4, 5 and 8 to adhere to, and invade INT-407 tissue culture cells, and to kill *C. elegans* was determined (Figure 6.5, Appendix 32 and Figure 6.6, Appendix 33). In the *C. elegans* model, *E. coli* 3950 containing all four plasmids killed the nematodes at a comparable rate to *E. coli* 3950 pCT (Figure 6.6), suggesting no association of these genes with the virulence of *E. coli* 3950. The ability of *S. Typhimurium* SL1344 rif^R containing pCT3 and pCT5 to adhere to and invade tissue culture cells was also unimpaired when compared to *S. Typhimurium* SL1344 rif^R pCT. However, the ability of *S. Typhimurium* SL1344 rif^R pCT4 and *S. Typhimurium* SL1344 rif^R pCT8 to invade the eukaryotic cells (INT-407 cells) was significantly reduced. *S. Typhimurium* SL1344 rif^R containing pCT3, pCT4, pCT5 and pCT6 (and *ISEcp1::aph*; chapter 4) also killed *C. elegans* at a slower rate than *S. Typhimurium* SL1344 rif^R pCT. On further analysis the rate of killing of these strains was comparable to that of *S. Typhimurium* SL1344 rif^R (without plasmid), it appears that carriage of wild-type pCT increases the ability of *S. Typhimurium* SL1344 rif^R pCT to cause infection in the *C. elegans* whereas carriage of the other pCT mutant plasmids results in a similar ability to cause infection in the *C. elegans* model as parental host strain *S. Typhimurium* SL1344 rif^R without plasmid. This might suggest that addition of *aph* onto pCT, or an affect of the recombination technique itself attenuates any small pathogenicity effects that wild-type pCT confers to the *S. Typhimurium* SL1344 rif^R host.

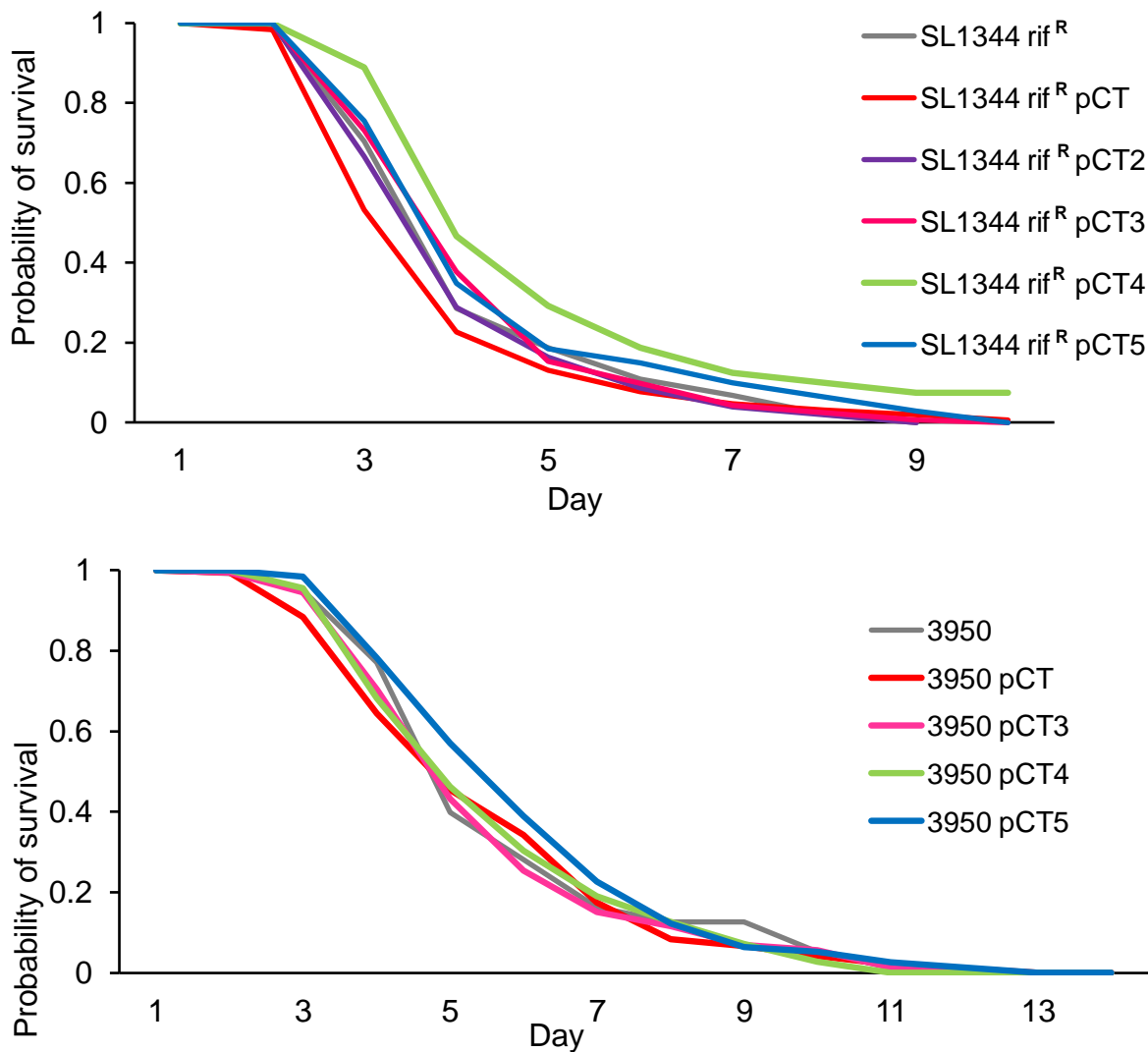
Figure 6.5. The ability of *S. Typhimurium* SL1344 rif^R containing wild-type pCT, pCT3, pCT4, pCT5 and pCT8 to adhere to and invade human intestinal cells



The number of *S. Typhimurium* SL1344 rif^R containing pCT3 pCT4, pCT5 or pCT8 able to adhere to (A) and invade (B) INT-407 human intestinal cell is represented as a percentage of the number of *S. Typhimurium* SL1344 rif^R pCT able to do so under test conditions (Appendix 32). Data are displayed as a mean of three separate experiments all performed with four technical repeats.

Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant are represented with an asterix.

Figure 6.6. The ability of bacterial hosts containing pCT3, pCT4, pCT5 and pCT8 to cause infection in *C. elegans*



Survival curves were constructed using a Kaplan Mier calculation showing the probability of *C. elegans* survival each day when fed continuously on the tested bacterial strains.

A significance difference was found in the killing rate between *S. Typhimurium* SL1344 rif^R pCT and *S. Typhimurium* SL1344 rif^R pCT3 (Sigma factor::*aph*) ($P = 0.023$), *S. Typhimurium* SL1344 rif^R pCT4 (*rci*::*aph*) ($P = <0.001$) and *S. Typhimurium* SL1344 rif^R pCT5 (*pilS*::*aph*) ($P = 0.0037$). A significant difference in killing rate was defined as a P value <0.05 (Appendix 33).

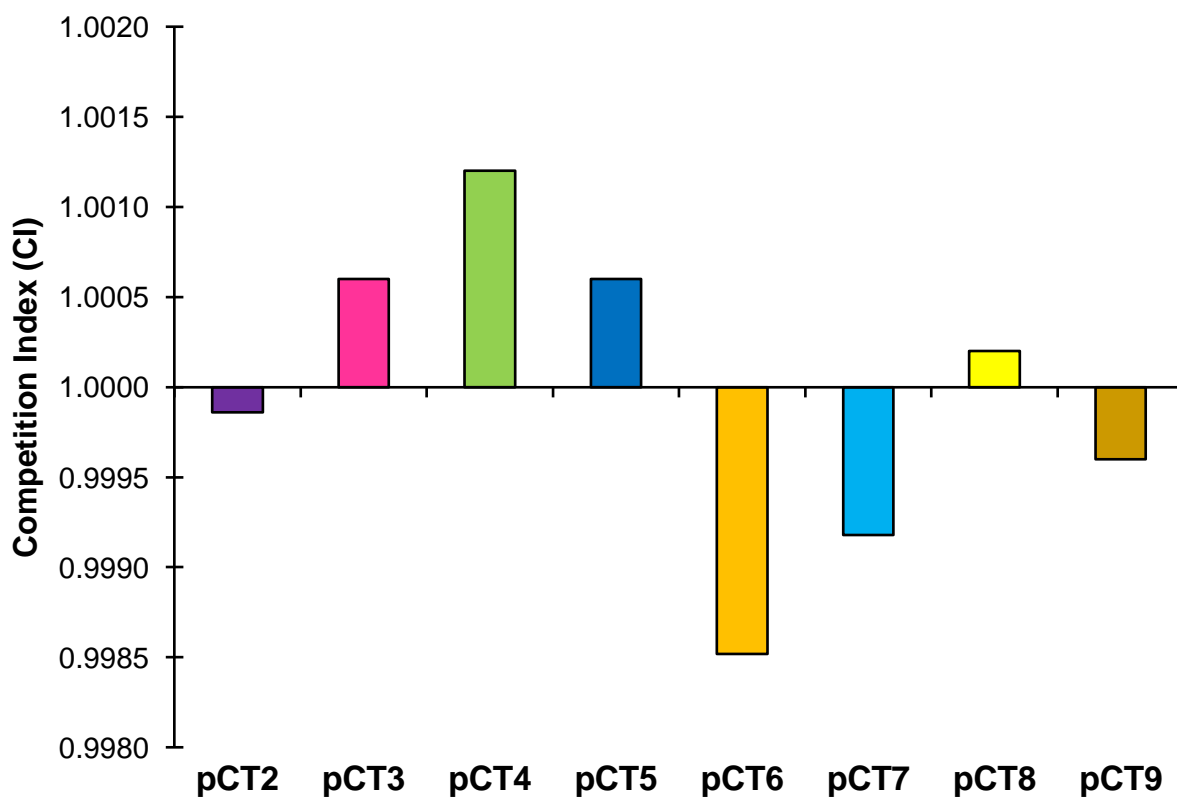
6.5 Competition of pCT3- 9 against wild-type pCT *in vitro*

To investigate whether inactivation of the genes on plasmids pCT3-9 had any effect on the plasmid's competitive fitness within an *in vitro* population, *E. coli* DH5 α containing plasmids pCT3-9 were directly competed in a 1:1 ratio with *E. coli* DH5 α pCT as in section 5.9. An *E. coli* DH5 α host was chosen for this experiment to allow assessment of all the mutant plasmids. pCT2 was used as a positive control as this plasmid was previously shown to remain in a 1:1 ratio during competition with pCT (chapter 5). All of the plasmid pairings remained in roughly equal proportions over the course of each 72 hour experiment and each plasmid mutant showed a competition index within 1.5×10^{-3} of 1 (Figure 6.7, Appendix 34). These data indicate that the genes inactivated in plasmids pCT2-9 had no direct effect on pCT competitive fitness *in vitro* in the short term.

6.6 Pair-wise competition between *E. coli* DH5 α and DH5 α pCT9 *in vitro*

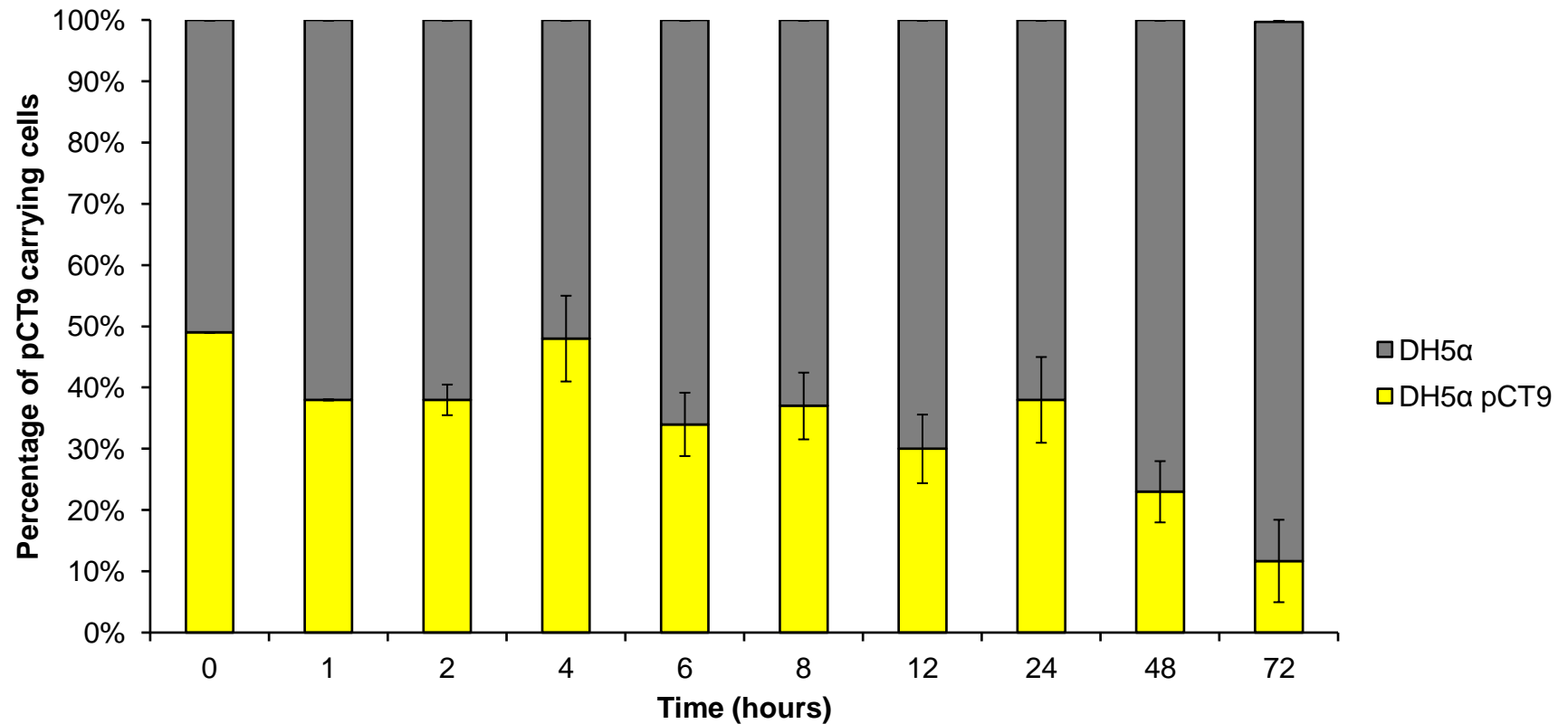
Previously in section 4.3.4, *E. coli* DH5 α pCT7 was shown to remain at a 1:1 ratio when co-cultured with its plasmid free parental strain. As pCT7 cannot transfer to plasmid free cells, and remains stable within the same time period in pure culture, it was hypothesised that pCT7 confers no fitness cost or benefit to the host cell. To investigate whether the inactivation of *pndACB* altered the stability of pCT7 and determine whether there is a role for *pndACB* in persistence, *E. coli* DH5 α pCT9 (*traXY::aph; pndACB::aph*) was competed in a 1:1 ratio with parental strain *E. coli* DH5 α on three separate occasions. Within 12 hours, the proportion of *E. coli* DH5 α pCT9 cells in the culture was 30%, and at 72 hours was 12% (Figure 6.8).

Figure 6.7. Competition Index of plasmids pCT3-9 when competed *in vitro* against wild-type pCT



Each pair-wise competition was conducted between *E. coli* DH5 α pCT and DH5 α containing the test plasmid *in vitro*. CI, competition index was calculated based on the mean values of at least three separate experiments (Appendix 34).

Figure 6.8. Pair-wise competitive growth *in vitro* between *E. coli* DH5 α and *E. coli* DH5 α pCT9 (*tra::aph*; *pndAC::cat*)



Competition index of DH5 α pCT9 at 12 hours (before first passage) = 0.9807, $P = <0.001$ (A significant change)*

Competition index of DH5 α pCT9 at 72 hours (after three passages) = 0.9859, $P = <0.001$ (A significant change)*

The percentage of bacteria carrying plasmid pCT9 over time was calculated when *E. coli* DH5 α +/- pCT9 were co-cultured in a 1:1 ratio *in vitro*. Cultures were passaged at hour 12, 24 and 48. The competition index was calculated from averages generated from at least three separate experiments.

As pCT9 remained stable in a pure culture *in vitro* under the same conditions, these data cannot be attributed to plasmid loss. Instead these data indicate that *E. coli* DH5 α pCT9 cells were outcompeted by the host alone over this short time period.

6.7 The role of pCT and the *pil* and *tra* locus in host biofilm formation and aggregation

The ability of a bacterial strain to aggregate or to form a biofilm can be considered a factor in its fitness or success; perhaps increasing its pathogenicity or broadening the range of environmental niches in which it can persist or thrive (Parsek and Singh, 2003; Pope *et al.*, 2010). The production of conjugation pili has been associated with the initiation, formation and stability of bacterial biofilms (Molin and Tolker-Nielsen, 2003). Therefore, the role of pCT and the production of pCT conjugation pili in biofilm formation and aggregation was investigated.

6.7.1 The ability of host strains containing pCT and pCT2-7 to form a biofilm

The biofilm formation of the four host strains *E. coli* DH5 α , *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R +/- pCT and the pCT mutants (pCT2-7) was investigated using the crystal violet biofilm assay, and by culturing these strains on agar containing Congo red to determine the production of *E. coli* and *Salmonella* biofilm components curli and cellulose.

6.7.1.1 *Crystal violet staining*

The natural veterinary *E. coli* isolate C159/11, and host strains *S. Typhimurium* SL1344 rif^R, *E. coli* J53-2 and *E. coli* 3950 formed poor biofilms in the presence or absence of both pCT and the pCT mutants (pCT2-7), revealing that the addition of pCT did not confer the ability to produce a biofilm to these host strains. *E. coli* 3950

pCT5 and *E. coli* 3950 pCT6 produced significantly more biofilm when compared to *E. coli* 3950 pCT. However, as the parental strain formed little biofilm it is unclear whether this small but consistent increase is a result of increased formation of a complex biofilm or just better adhesion (Figure 6.9, Appendix 35).

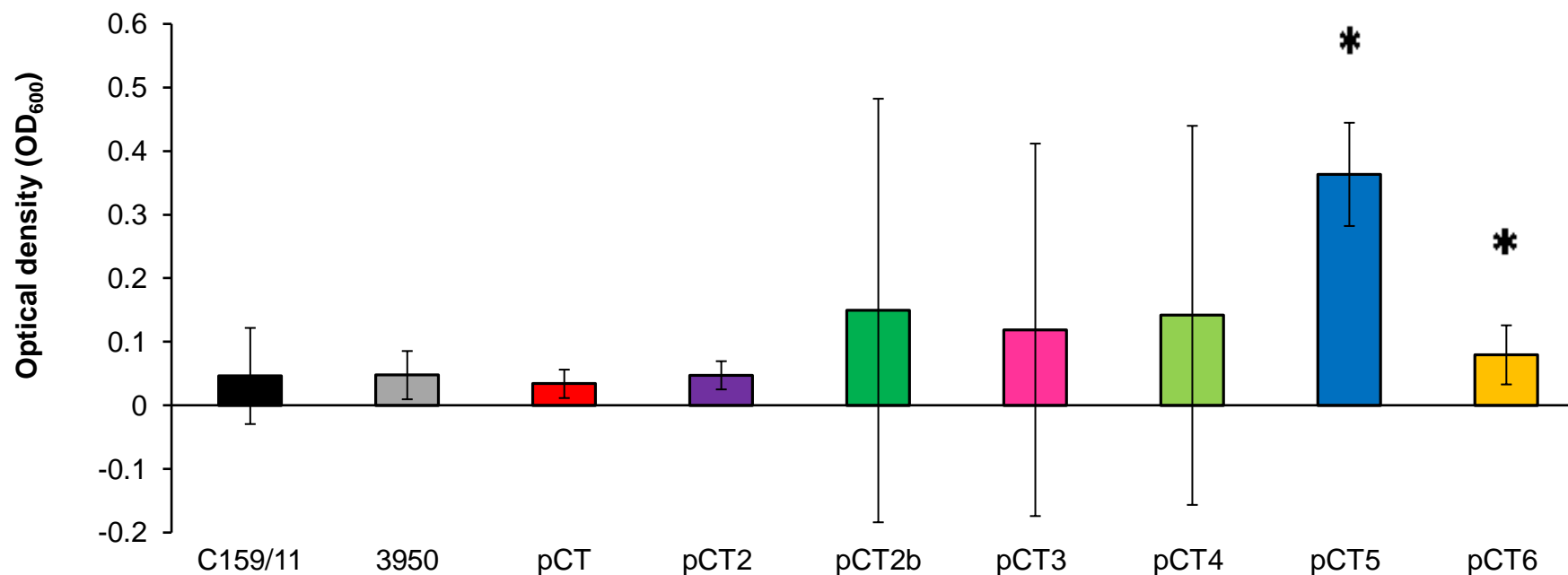
E. coli DH5 α formed a significant biofilm and the addition of pCT, pCT2 and pCT4-7 did not affect its ability to do so (Figure 6.10, Appendix 35). *E. coli* DH5 α pCT3 (Sigma factor::*aph*) had a significantly increased ability to biofilm and *E. coli* DH5 α pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) appeared to have a reduced biofilming ability. As this effect was not seen in *E. coli* DH5 α pCT2 (*bla*_{CTX-M-14}::*aph*), the result is unlikely to be due to the inactivation of *bla*_{CTX-M-14}, and may be due to an effect of the production of GFP.

Plasmids pCT and pCT2-6 were also transferred via conjugation to *S. Typhimurium* strain 14028S (L828 rif^R) a strain which produces high levels of biofilm, and the ability of these new strains to biofilm was measured. Addition of all seven plasmids had no effect on the ability of 14028S to biofilm. Therefore, pCT does not appear to increase or decrease the ability of a bacterium to form biofilms in strains that already do so, and changes in biofilm formation are not likely to result from the carriage of pCT.

6.7.1.2 *The production of curli and cellulose using Congo red staining*

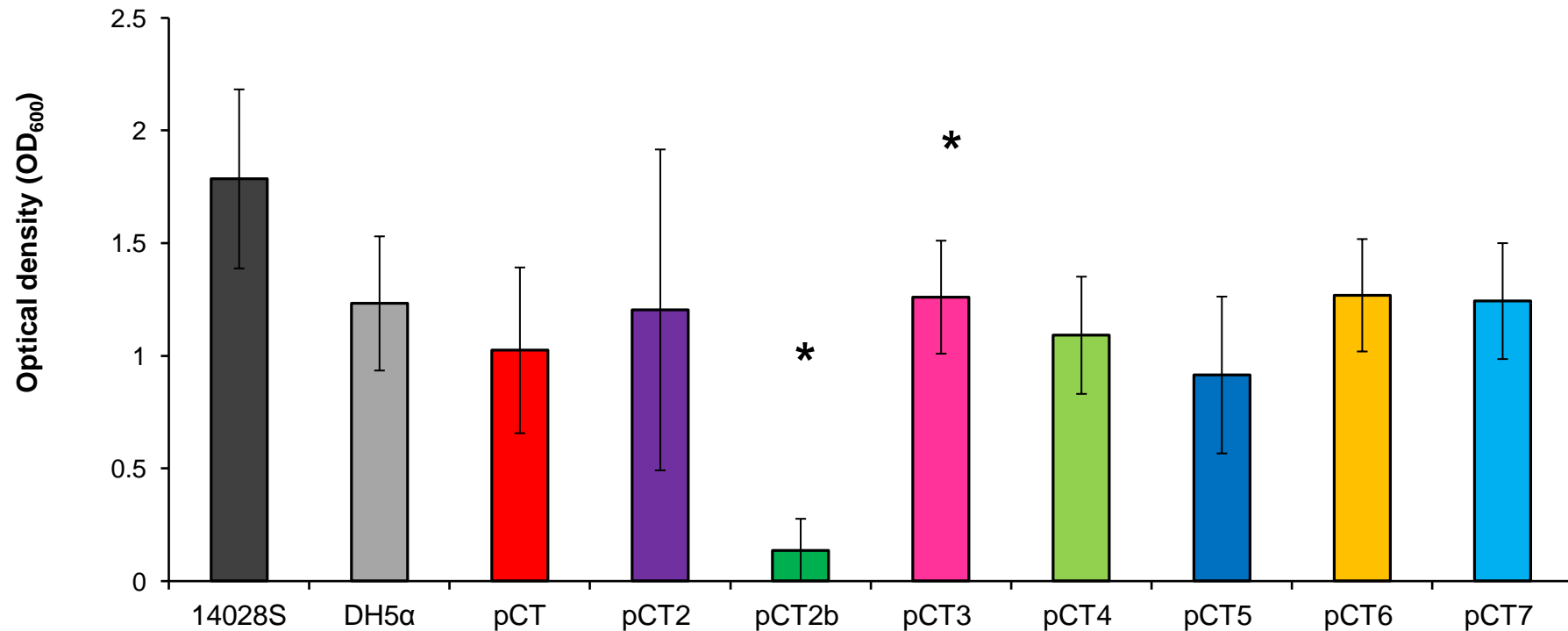
Biofilm formation was also investigated by observing the colony morphology of each host strain (+/- pCT and the pCT plasmid mutants) when grown on LB agar without salt, supplemented with 40 mg/L of Congo red (Figure 6.11). All *E. coli* J53-2 colonies were of a pink, dry and smooth morphology suggesting low production of curli and cellulose; addition of the pCT plasmids had no effect in this strain. Similarly, the addition of pCT and pCT2-6 had no effect on colony morphology

Figure 6.9. Biofilm formation of C159/11 and 3950 containing the plasmids pCT and pCT2-6



The ability of each bacterial strain to produce biofilm was measured by staining each biofilm with crystal violet and using optical density (OD₆₀₀) to quantify each biofilm. Optical density values of four technical repeats and at least four biological repeats were compared using a student T test where a *P* value of <0.05 from a Student's T-test were considered statistically significant. Those strains found to have significantly different values compared to strains containing plasmid pCT are denoted with an asterisk (Appendix 35).

Figure 6.10. Biofilm formation of DH5 α containing the plasmids pCT and pCT2-7



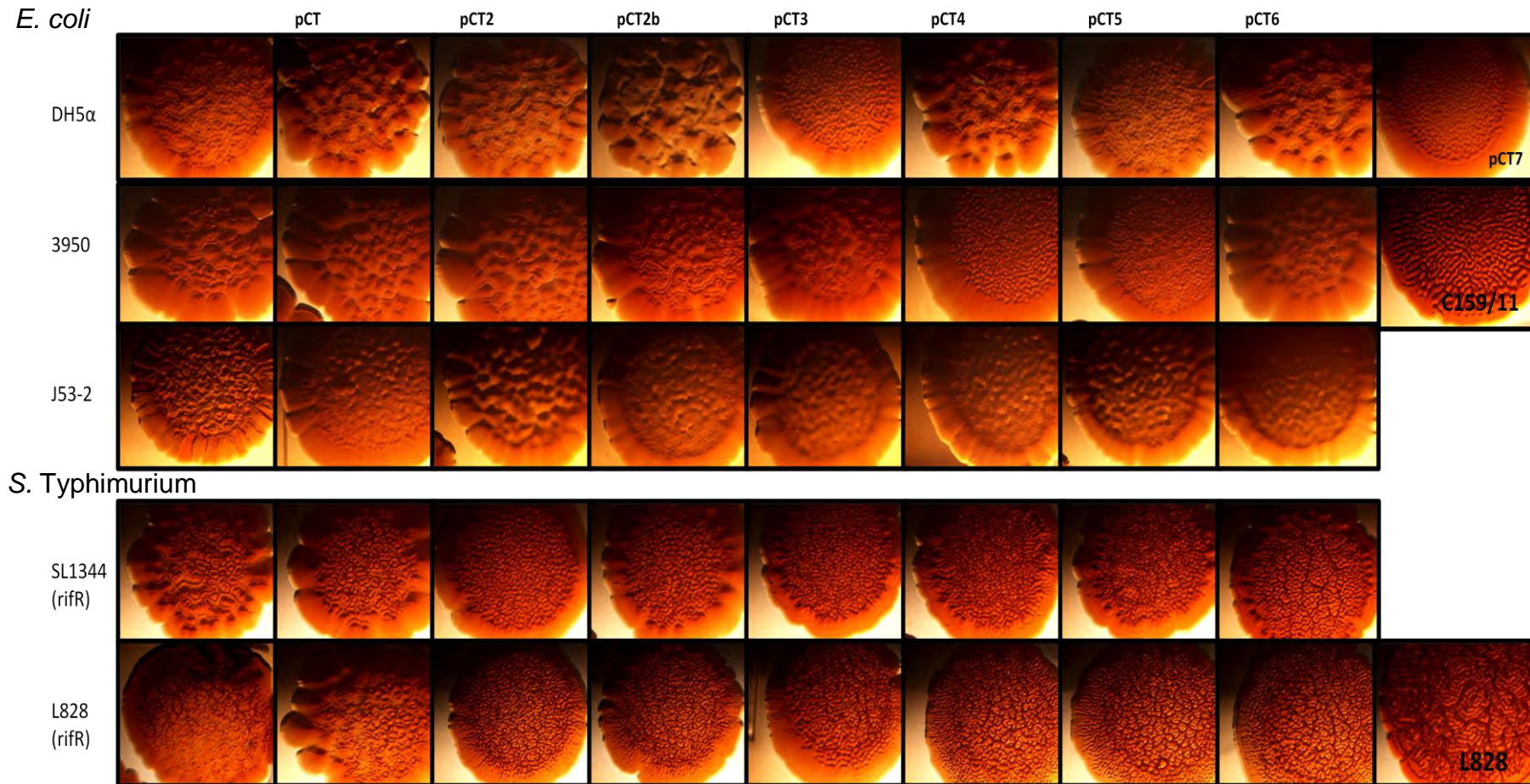
The ability of each bacterial strain to produce biofilm was measured by staining each biofilm with crystal violet and using optical density (OD₆₀₀) to quantify each biofilm. Optical density values of four technical repeats and at least four biological repeats were compared using a student T test. Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant. Those strains found to have significantly different values compared to strains containing plasmid pCT are denoted with an asterisk (Appendix 35). *S. Typhimurium* 14028S was used as a positive control.

of the *S. Typhimurium* strains SL1344 *rif*^R and 14028S *rif*^R which were red, dry and rough (rdar) indicating the production of curli and cellulose. Veterinary strain *E. coli* C159/11 had a classic rdar phenotype, however *E. coli* 3950 (pCT cured derivative of C159/11) and *E. coli* 3950 containing pCT and pCT2-6 had a pinker and smoother morphology, particularly *E. coli* 3950 pCT4 (*rci::aph*) and *E. coli* 3950 pCT5 (*pilS::aph*). The *E. coli* DH5α strains also differed in their appearance, as *E. coli* DH5α pCT3 (Sigma factor::*aph*) and *E. coli* DH5α pCT7 (*traXY::aph*) appeared smoother than other strains. However, it was noted that no change in morphology due to the presence of any particular plasmid was seen consistently in all hosts (Figure 6.11).

6.7.2 Aggregation of bacterial hosts containing plasmids pCT and pCT2-6

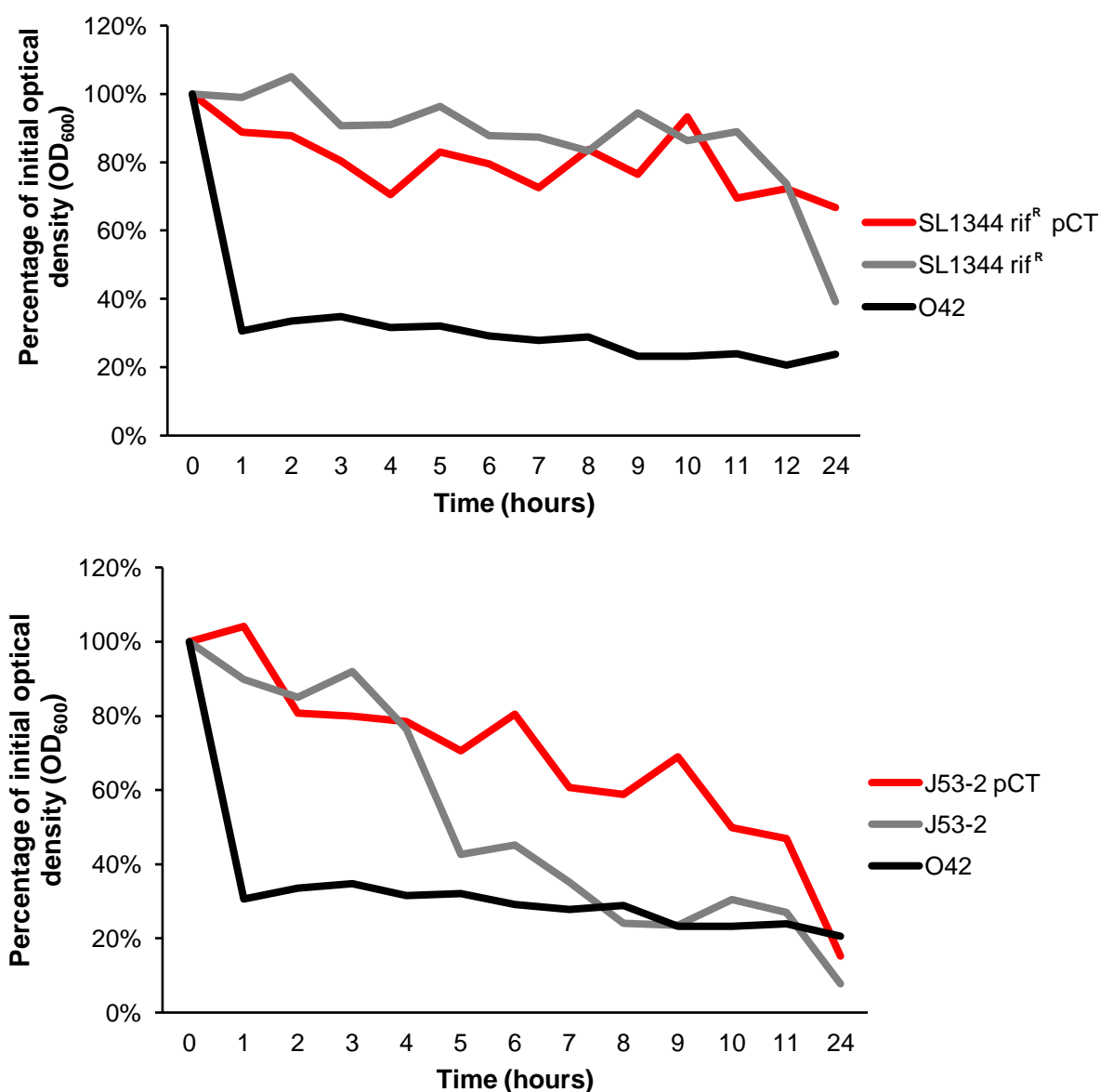
To determine whether the addition of pCT and the pCT mutants pCT2-6 affected the ability of host strains *S. Typhimurium* SL1344 *rif*^R and *E. coli* J53-2 to aggregate, settle assays were performed. Overnight cultures of each strain were kept stationary for 24 hours. During the first 12 hours and at 24 hours a sample was removed from the top of each culture and the optical density was measured. Strains able to aggregate, such as positive control *E. coli* 042, showed a rapid reduction in optical density as the cells aggregated within the broth. The addition of pCT, the pCT *pil* locus mutants (pCT4 and pCT5) and pCT2, pCT3 and pCT6 had no effect on the host strain's ability to aggregate (Figure 6.12, Figure 6.13 and Figure 6.14, Appendix 36). These data suggest that pCT does not confer either an increased or decreased aggregative ability to host strains.

Figure 6.11. Colony morphology of bacterial hosts strains containing plasmid pCT and plasmid mutants (pCT2-7) grown on LB agar containing Congo red



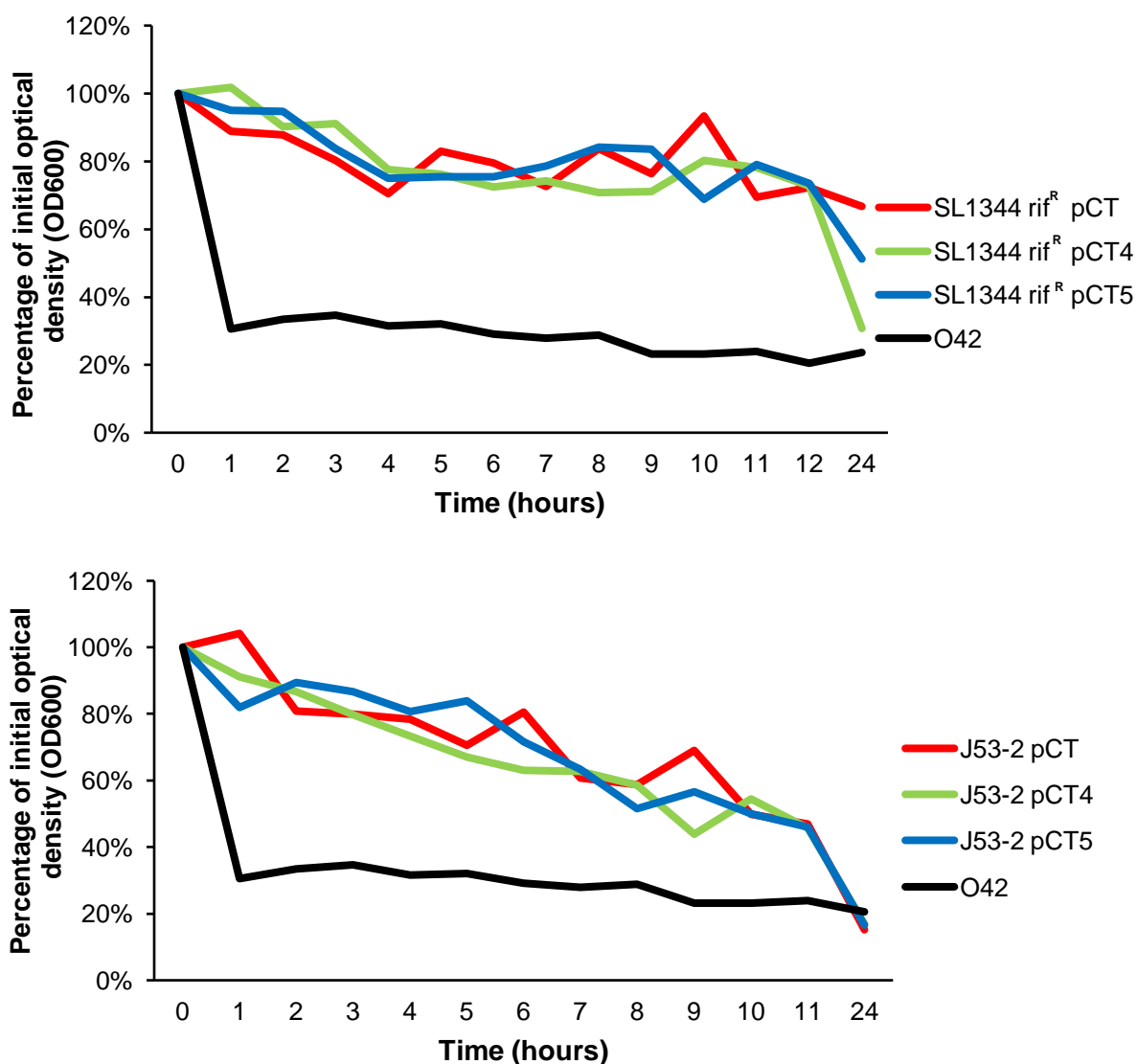
Colony morphology: red, dry and rough colonies (rdar) indicate curli and cellulose production; Brown, dry and rough (bdar) colonies indicate curli production without cellulose; Pink, dry and rough (pdar) colonies indicate cellulose production without curli and white and smooth colonies (saw) indicate a lack of both curli and cellulose production.

Figure 6.12. The aggregative ability of *S. Typhimurium* SL1344 rif^R and *E. coli* J53-2 +/- pCT



Bacterial strains were left in a stationary culture over a 24 hour period. The optical density of these cultures was monitored over this time and expressed as a percentage of the starting optical density. A drop in optical density suggested bacterial cell aggregation. Average optical density changes of strains +/- pCT were compared using a Student's T-test. Values returning a *P* value of <0.05 were considered statistically significant and are denoted with an astrix (Appendix 36) *E. coli* O42 was used as a positive control.

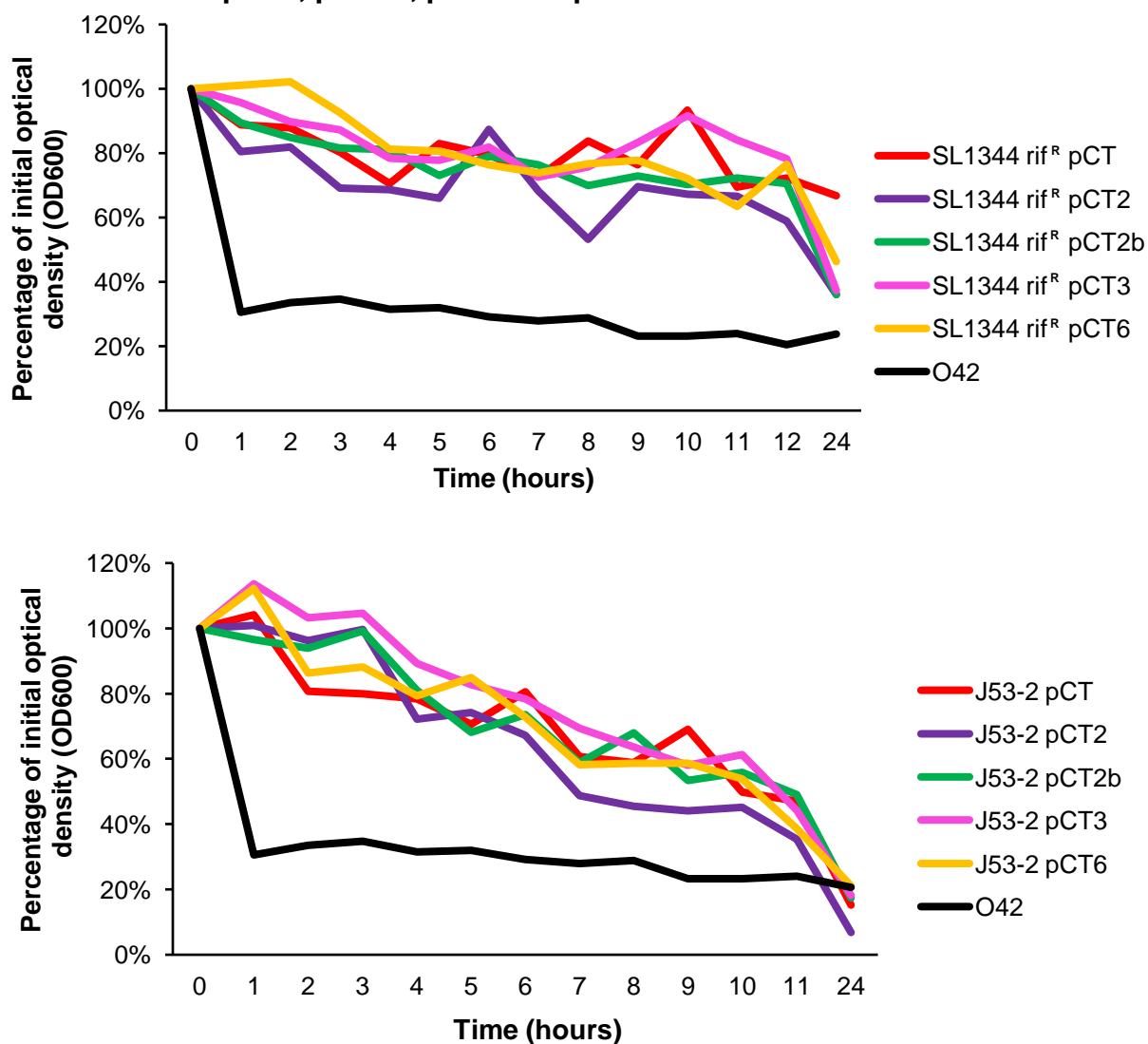
Figure 6.13. The aggregative ability of *S. Typhimurium* SL1344 rif^R and *E. coli* J53-2 +/- pCT4 and pCT5



Bacterial strains were left in a stationary culture over a 24 hour period. The optical density of these cultures was monitored over this time and expressed as a percentage of the starting optical density. A drop in optical density suggested bacterial cell aggregation. Average optical density changes of strains containing pCT4 and pCT5 were compared to strains containing pCT using a Student's T test. Values returning a *P* value of <0.05 were considered statistically significant and are denoted with an asterix (Appendix 36)

E. coli 042 was used as a positive control.

Figure 6.14. Aggregative ability of *S. Typhimurium* SL1344 rif^R and *E. coli* J53-2 +/- pCT2, pCT2b, pCT3 and pCT6



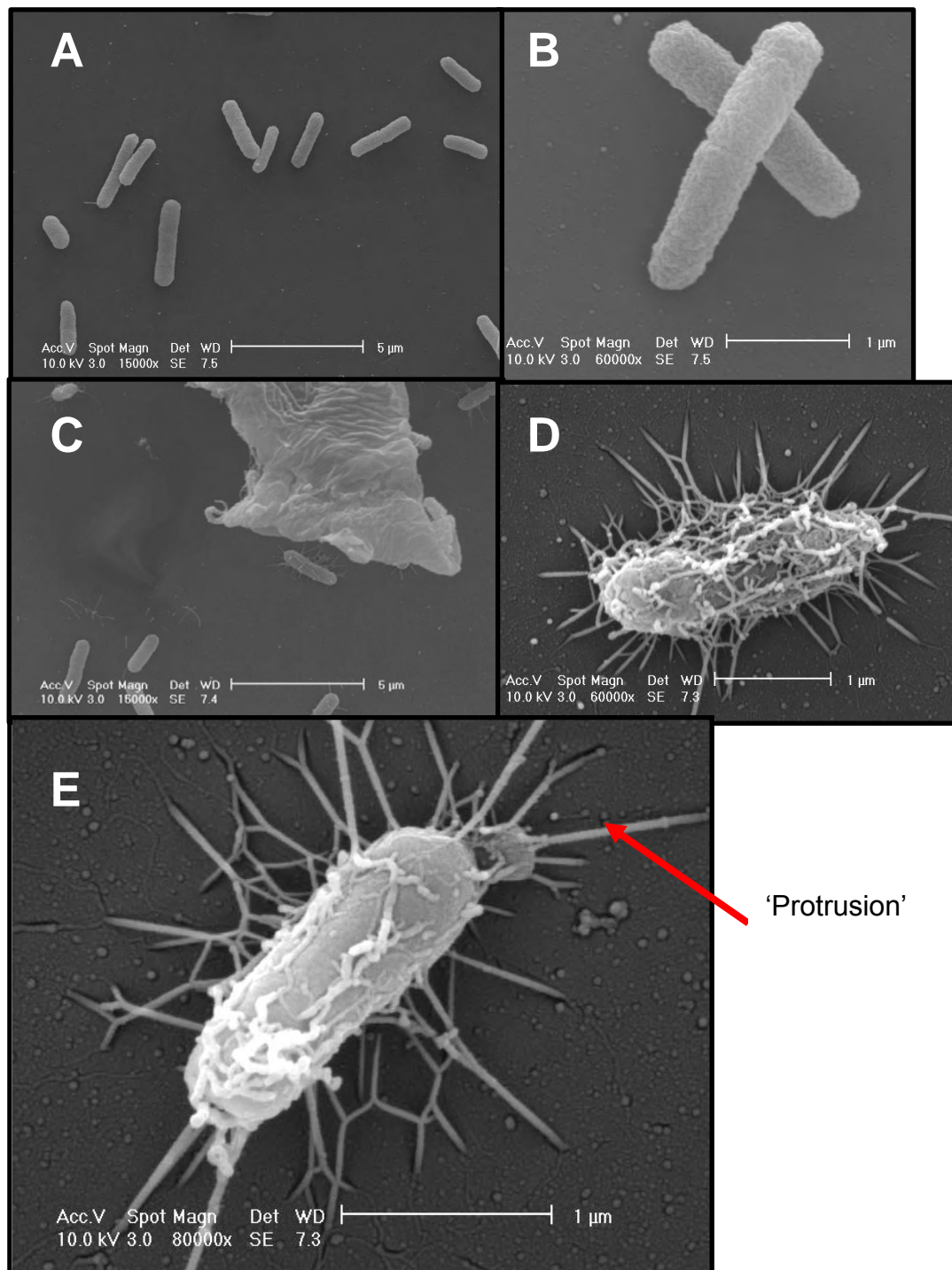
Bacterial strains were left in a stationary culture over a 24 hour period. The optical density of these cultures was monitored over this time and expressed as a percentage of the starting optical density. A drop in optical density suggested bacterial cell aggregation. Average optical density changes of strains containing pCT2, pCT2b, pCT3 and pCT6 were compared to strains containing pCT using a Student's T-test. Values returning a *P* value of <0.05 were considered statistically significant and are denoted with an asterix (Appendix 36) *E. coli* O42 was used as a positive control.

6.7.3 Visualisation of cells using Scanning Electron Microscopy (SEM)

To investigate the effects of adding pCT, and inactivating pCT genes *rci*, *pilS* and *traXY* on *E. coli* DH5 α and *S. Typhimurium* SL1344 rif^R host strain pili production, bacterial host strains were grown in LB broth to late-logarithmic phase, and overnight on an LB agar plate, and visualised using scanning electron microscopy. Visualisation of cells at magnification of x 15,000 to x 80,000 consistently revealed two distinct cell morphologies named in this study as ‘smooth cells’ and ‘cells with protrusions’ (Figure 6.15). Both cell types were enumerated for each strain (Table 6.2). The cells taken from colonies grown on agar plates were of the smooth morphology. *E. coli* DH5 α and *S. Typhimurium* SL1344 rif^R cells (without pCT) grown in broth were also smooth in appearance. However, *E. coli* DH5 α pCT and *S. Typhimurium* SL1344 rif^R pCT cells consists of both morphologies (52% and 11% showing protrusions, respectively) suggesting a direct association between the presence of pCT and cell protrusions (Figure 6.15, Figure 6.16). Strain *S. Typhimurium* SL1344 rif^R pCT4 (*rci::aph*) had a similar proportion of smooth cells and cells with protrusions to *S. Typhimurium* SL1344 rif^R pCT (13%) but *S. Typhimurium* SL1344 rif^R pCT5 (*pilS::aph*) had no cells with protrusions.

E. coli DH5 α containing pilus mutant plasmids pCT5 and pCT7 also had a reduced number of cells with protrusions compared to *E. coli* DH5 α pCT, although a few were still present. While these observations, particularly in *S. Typhimurium* SL1344 rif^R suggest that the protrusion morphology is linked to pilus formation, the *E. coli* DH5 α pCT5 (*pilS::aph*) and pCT7 (*traXY::aph*) cells with protrusions of suggests that this is may not be a direct association. Additional observations were that *E. coli* DH5 α pCT7 (*traXY::aph*) cells grown on agar were connected by irregular chain like structures,

Figure 6.15. Scanning electron microscopy images of DH5 α +/- pCT from LB broth



All images were taken using scanning electron microscopy (SEM). Cells were prepared by growing strains in LB broth to late-logarithmic phase and then fixed.

Panels A and B show *E. coli* DH5 α cells without plasmid pCT showing the smooth morphology

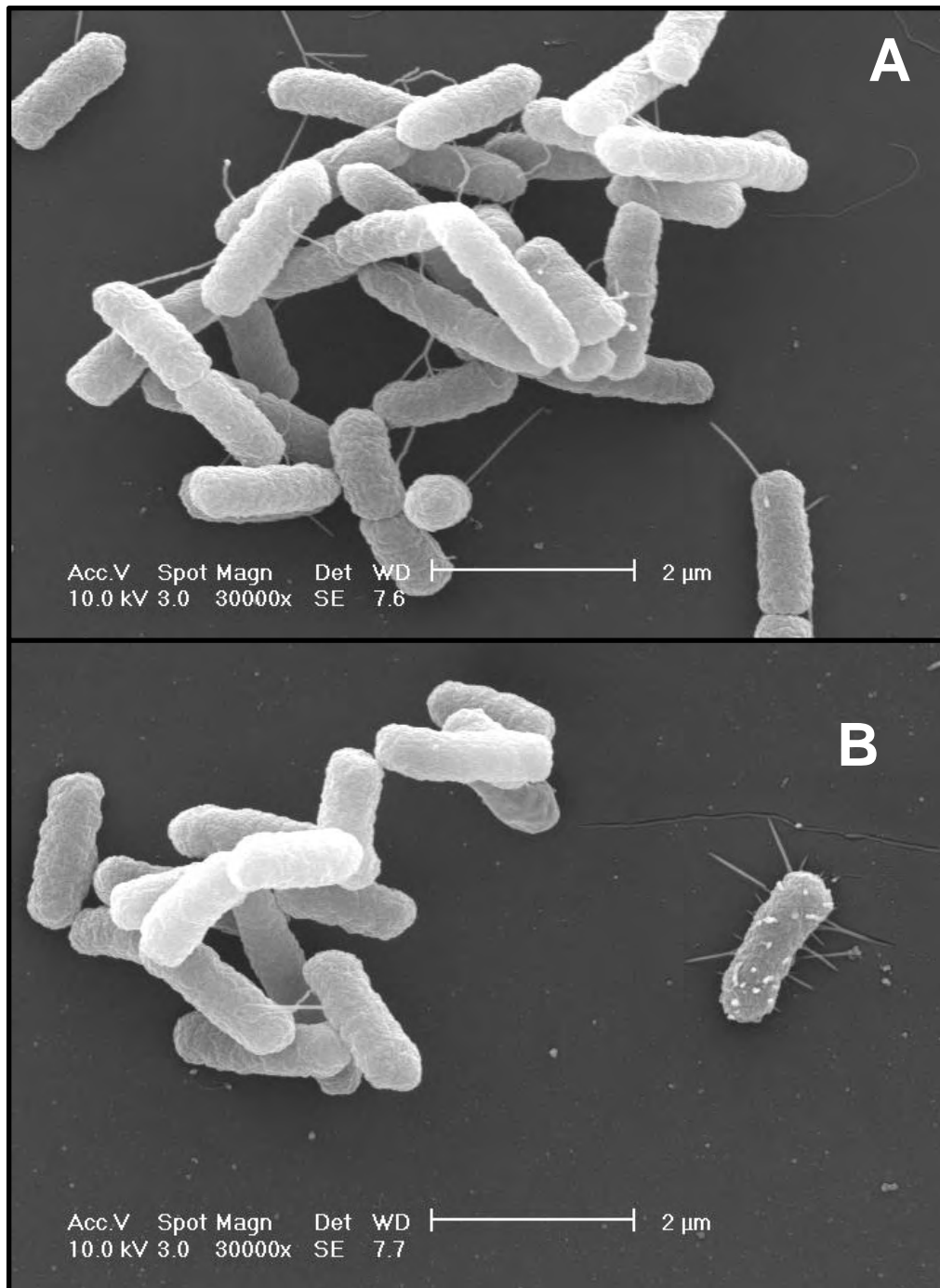
Panels C, D and E show *E. coli* DH5 α pCT cells with protrusions seen in ~50% of DH5 α cells containing pCT but in no cells which do not carry plasmid pCT.

Table 6.2. The proportion of cells with protrusions visualised using scanning electron microscopy

| Cell Prep | Species | Lab code | Strain | Number of cell examined | Number of cells with protrusions | Percentage (%) |
|-----------|-----------------------|----------------|---|-------------------------|----------------------------------|----------------|
| Broth | <i>E. coli</i> | I753 | C159/11 | 600 | 0 | 0 |
| | | I825 | DH5α | 300 | 0 | 0 |
| | | I755 | DH5α pCT | 200 | 104 | 52 |
| | | I829 | DH5α pCT5 (<i>pilS::aph</i>) | 200 | 10 | 5 |
| | | I846 | DH5α pCT7 (<i>traXY::aph</i>) | 200 | 8 | 4 |
| | <i>S. Typhimurium</i> | L1078 | SL1344 rif ^R | 300 | 0 | 0 |
| | | L1079 | SL1344 rif ^R pCT | 200 | 22 | 11 |
| | | L1259 | SL1344 rif ^R pCT4 (<i>rci::aph</i>) | 200 | 26 | 13 |
| | | L1260 | SL1344 rif ^R pCT5 (<i>pilS::aph</i>) | 200 | 0 | 0 |
| | Agar | <i>E. coli</i> | I753 | C159/11 | 200 | 0 |
| I825 | | | DH5α | 200 | 0 | 0 |
| I755 | | | DH5α pCT | 200 | 0 | 0 |
| I829 | | | DH5α pCT5 (<i>pilS::aph</i>) | 200 | 0 | 0 |
| I846 | | | DH5α pCT7 (<i>traXY::aph</i>) | 200 | 0 | 0 |

The effect of pCT7 on cell morphology could not be observed in *S. Typhimurium* SL1344 rif^R as conjugation and transformation of pCT7 into this strain was unsuccessful. The effect of pCT4 on cell morphology was visualised in host strain *S. Typhimurium* SL1344 rif^R as inactivation of *rci* modified the susceptibility of this host strain to cefotaxime and reduced the pCT conjugation rate to this recipient. The same effects were not observed in *E. coli* host strain DH5α.

Figure 6.16. SEM images of *S. Typhimurium* SL1344 rif^R cells +/- pCT from LB broth



All images were taken using scanning electron microscopy (SEM). Cells were prepared by growing strains in LB broth to late-logarithmic phase and then fixed.

Panel A shows *S. Typhimurium* SL1344 rif^R cells without plasmid pCT showing the smooth morphology

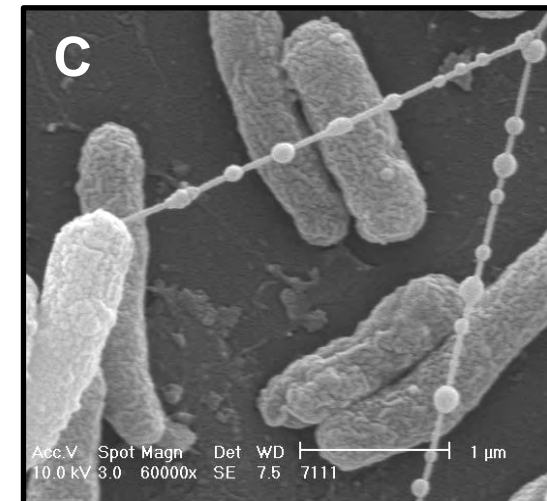
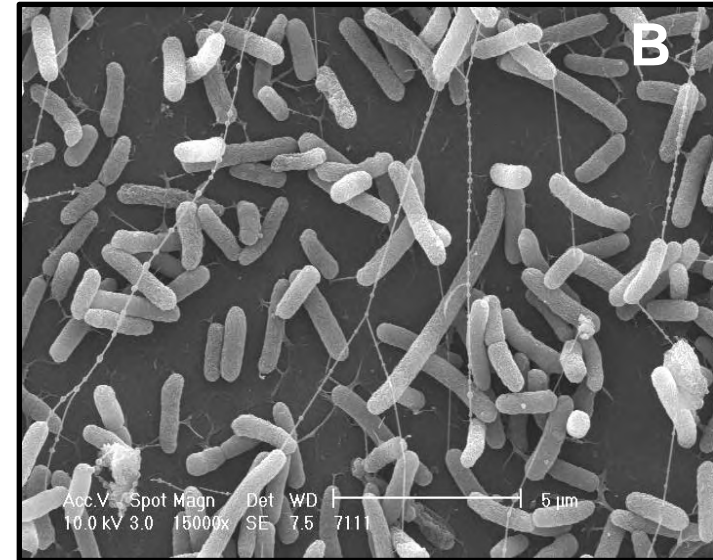
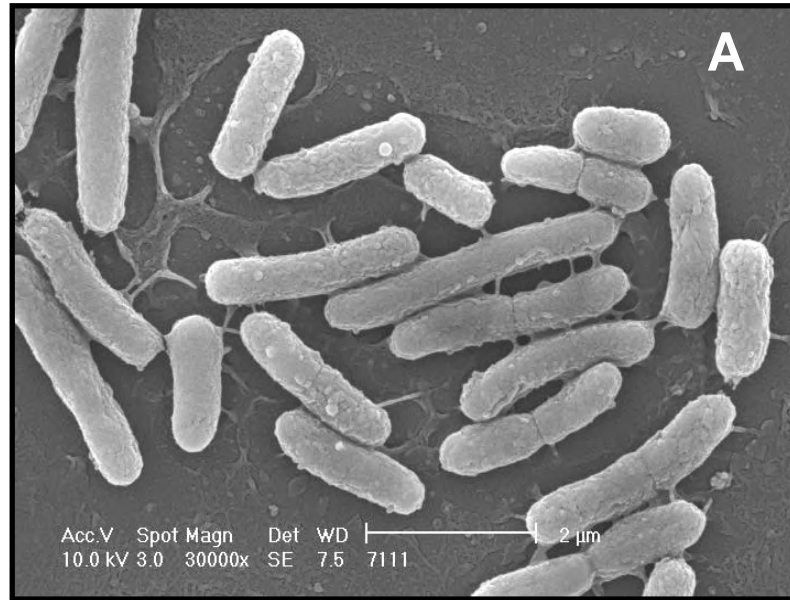
Panel B shows *S. Typhimurium* SL1344 rif^R pCT cells, 1/15 showing the protrusions.

not seen with any of the other strains (Figure 6.17). As pCT7 had an inactivated *tra* locus component, these chains may be the result of a misassembled, but still produced, thick pilus. *E. coli* C159/11 (pCT) grown on agar and in broth were also visualised; this revealed no cells with protrusions in either growth condition (Figure 6.18).

6.8 Discussion

Data presented in section 5.9 showed that in the absence of antibiotic pressure, resistance gene *bla*_{CTX-M-14} conferred neither a fitness cost nor benefit to host strains *E. coli* DH5 α and *E. coli* 3950. Plasmid pCT itself neither increased nor decreased the fitness of the host bacterium, was stably maintained within all daughter cells and easily transferred by conjugation. These data suggest that factors encoded within the pCT backbone may contribute to the evolutionary ‘fitness’ of this plasmid. Therefore, understanding of the pCT DNA sequence and annotation (Chapter 3) was used to select and systematically inactivate chosen genes, in order to assess their functional genomics and their contribution to the ‘success’ of pCT. The lack of similarity with other sigma factors such as SigZ encoded by IncI plasmid R64, and the absence of available data on plasmid borne sigma factors made pCT_066 an interesting candidate for investigation. Inactivation of the pCT putative sigma factor (pCT3) had no effect on host growth, either in pure culture or when competed with bacteria containing wild-type pCT; the ability of host bacteria to cause infection, susceptibility to β -lactams, or the ability of pCT to persist. A small but consistent non statistically significant increase in the conjugation frequency of pCT3 compared to pCT was seen in all the conditions tested.

Figure 6.17. Scanning electron microscopy images of *E. coli* DH5 α pCT and *E. coli* DH5 α pCT7 cells from LB agar

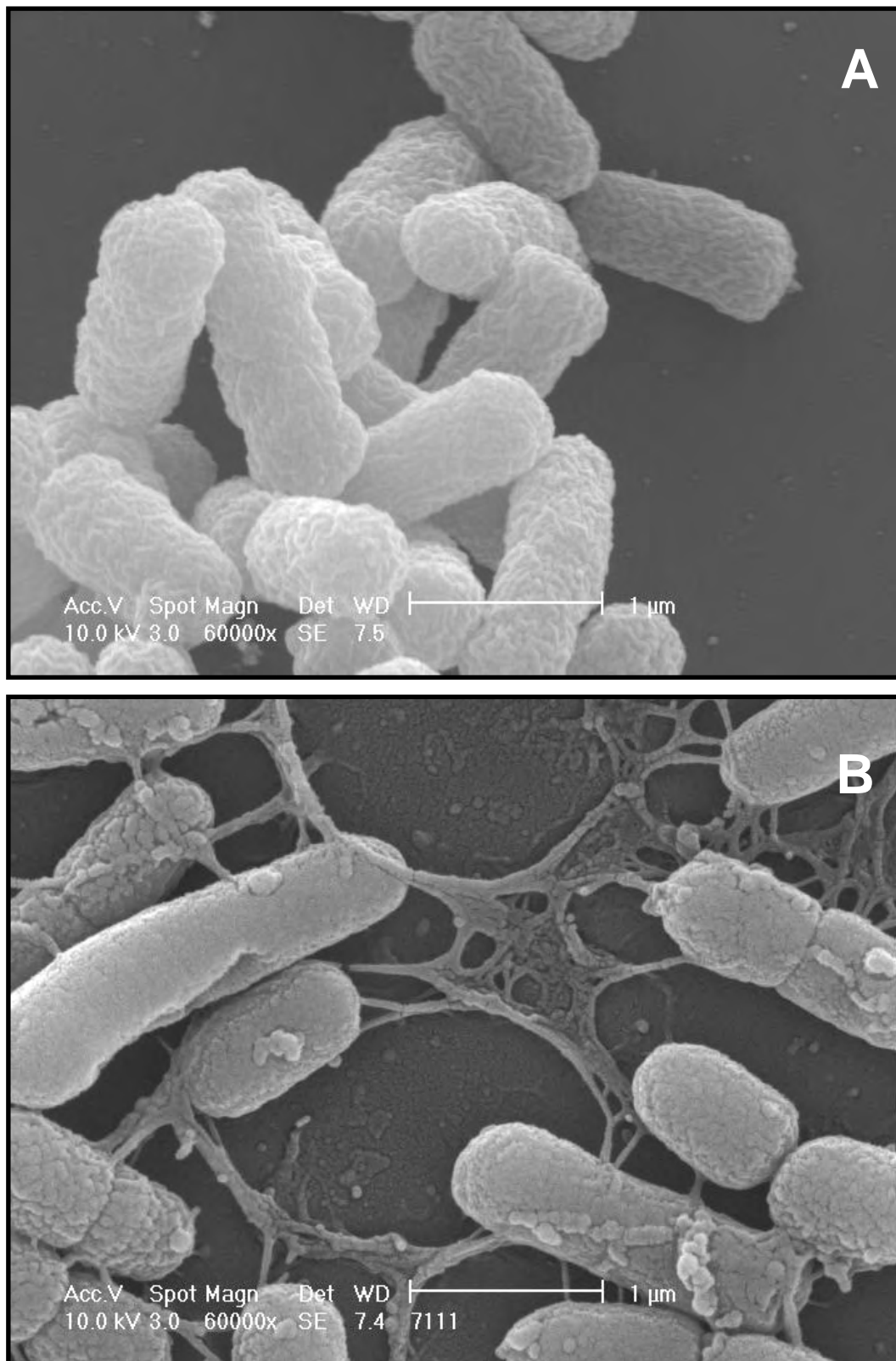


All images were taken using scanning electron microscopy (SEM). Cells were prepared by growing strains on LB agar overnight, resuspending in PBS and then fixing them onto glass.

Panel A shows *E. coli* DH5 α pCTs cells with a smooth morphology.

Panels B and C show *E. coli* DH5 α pCT7 cells(*traXY::aph*) showing regular chain like structures between cells.

Figure 6.18. SEM images of *E. coli* C159/11 from broth and agar



Panel A shows *E. coli* C159/11 (pCT) cells grown in LB broth
Panel B shows *E. coli* C159/11 (pCT) cells grown on LB agar
Both show smooth cells without protrusions despite carrying plasmid pCT

Although there is little reference to the direct control of conjugation by sigma factors within the literature, a Sigma 70 binding site has been identified directly upstream of a pili biogenesis regulatory gene (*traA*) in plasmid R64 (Kim *et al.*, 1993), suggesting that there may be some association in R64. Inactivation of the pCT putative sigma factor gene also significantly increased the ability of *E. coli* DH5 α to biofilm. Chromosomally encoded sigma factors can influence bacterial biofilm formation and the expression of curli biosynthetic genes, for example, in response to environmental changes or stressors (Gualdi *et al.*, 2008). Therefore it may be that the small effects of inactivating the pCT putative sigma factor observed during my *in vitro* experiments, may be amplified in a natural and stressful environment (Stockwell and Loper, 2005). Similarly, non-essential extra-cytoplasmic function sigma factors (ECF) encoded by *Bacillus subtilis* facilitate a more rapid cellular response to stress (Asai *et al.*, 2008). If their non essential nature has allowed ECF sigma factors to become mobile (for example on conjugative plasmids), and to confer an advantage in stressful environments it is conceivable that this would increase the fitness and persistence of these plasmids in certain conditions, which were not necessarily reproduced in the laboratory in the present study. Host bacterial chromosomally encoded sigma factors are essential for plasmid replication (Wado *et al.*, 1987), and their availability can be a limiting factor in plasmid DNA synthesis and copy number control (Schmidt *et al.*, 1996). Therefore, provision of a plasmid encoded sigma factor has the potential to alleviate reliance on the host cell σ 70 expression, allow better copy number control, or widen the plasmid host range. Although no clear phenotype was attributed to the pCT sigma factor, a subtler effect of plasmid encoded sigma factors on the ability of host bacteria to biofilm, on pili expression or copy number control is possible.

To investigate the contribution of thick (*tra*) and thin (*pil*) pili on pCT dissemination and host fitness, the effects of inactivating the major structural proteins of each pilus (*traY* and *pilS*) were assessed. Inactivation of *traY* prevented all pCT transfer as previously described for R64 (Komano *et al.*, 2000). These data confirm the role of the thick pilus in pCT conjugation in liquid and on a surface. The inactivation of thin pilus gene, *pilS*, reduced the frequency of pCT conjugation in liquid to recipients *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R but did not preclude transfer. These data suggest that the thin pilus is not required for pCT conjugation, but does increase the frequency with which pCT can conjugate to certain recipient hosts. As inactivation of *pilS* had no effect on pCT transfer on a filter to *E. coli* recipients, the role of the thin pilus in conjugation on a solid surface is less clear. However, inactivation of *pilS* and *rci* (which determines the epitope found at the tip of the thin pilus) reduced the rate of transfer to recipient *S. Typhimurium* SL1344 rif^R. These data indicate that the thin pilus may be more important for conjugation into particular host strains, and that an active *rci* gene may allow variation in the bacterial host range.

Inactivation of both conjugation systems had no significant effect on host generation times, on the competitive index of the plasmid when placed in a 1:1 ratio with wild-type pCT, or on the ability of pCT to persist within host bacteria. Disruption of the pCT *pil* loci also had no effect on the host bacterium's ability to adhere to eukaryotic cells, to aggregate or to form a biofilm. These results are in concordance with Srimanote *et. al.* (2002) who found that the *pil* locus of IncI plasmid pO113 was not responsible for bacterial adhesion to eukaryotic cells. However, they are inconsistent with the finding of Dudley *et al.* (2006) who showed that inactivation of *pilS* on IncK plasmid pSERB1 (potentially a pCT-like plasmid) reduced host adherence to cells by

75% and reduced biofilm formation by up to 50%, strongly suggesting an adhesive role for the thin pilus. As pCT, pO113 and pSERB1 are very similar plasmids sharing analogous *pil* loci, it may be that the differing adhesion abilities conferred are due to the different bacterial host strains (pO113 host strain O113:H21 STEC 98NK2 and pSERB1 host EAEC C1096) or interactions between other plasmid and host factors. If this were the case, the pCT *pil* locus may confer increased adhesion or biofilm forming ability to STEC or EAEC strains. Dudley *et al.*, (2006) also showed using scanning electron microscopy that most of the EAEC C1096 cells containing wild-type plasmid pSERB1 displayed 'surface pili'. These appear very similar to the structures named in my study as 'protrusions'. In bacterial cells containing pSERB1 with an inactivated *pilS* a similar number of protrusion bearing cells were seen as in *E. coli* DH5 α pCT5 (*pilS::aph*), suggesting that the cell morphology observed in my study is reproducible and applicable to other similar plasmids. Dudley *et al.*, concluded that the structures were a direct result of *pil* locus expression. However, my study shows a more complex picture, as inactivation of pCT *traXY* (pCT7) resulted in a similar reduction of bacterial cells expressing protrusions as inactivation of pCT *pilS* (pCT5). The decrease, but not absence, of protrusion expressing *E. coli* DH5 α pCT5 and *E. coli* DH5 α pCT7 is also hard to explain if the structures are directly related to pili expression. Further study of the literature did not reveal any alternative convincing suggestions as to what the protrusions may be; other suggestions included bacterial nanotubules (Sanchez, 2011), glycocalyx (capsule structures) (Morck *et al.*, 1987) and tubular spinae (Bayer and Easterbrook, 1991). Once again the production of protrusions appears to be host specific (*E. coli* C159/11 cells did not produce protrusions despite containing pCT), and the effect was also

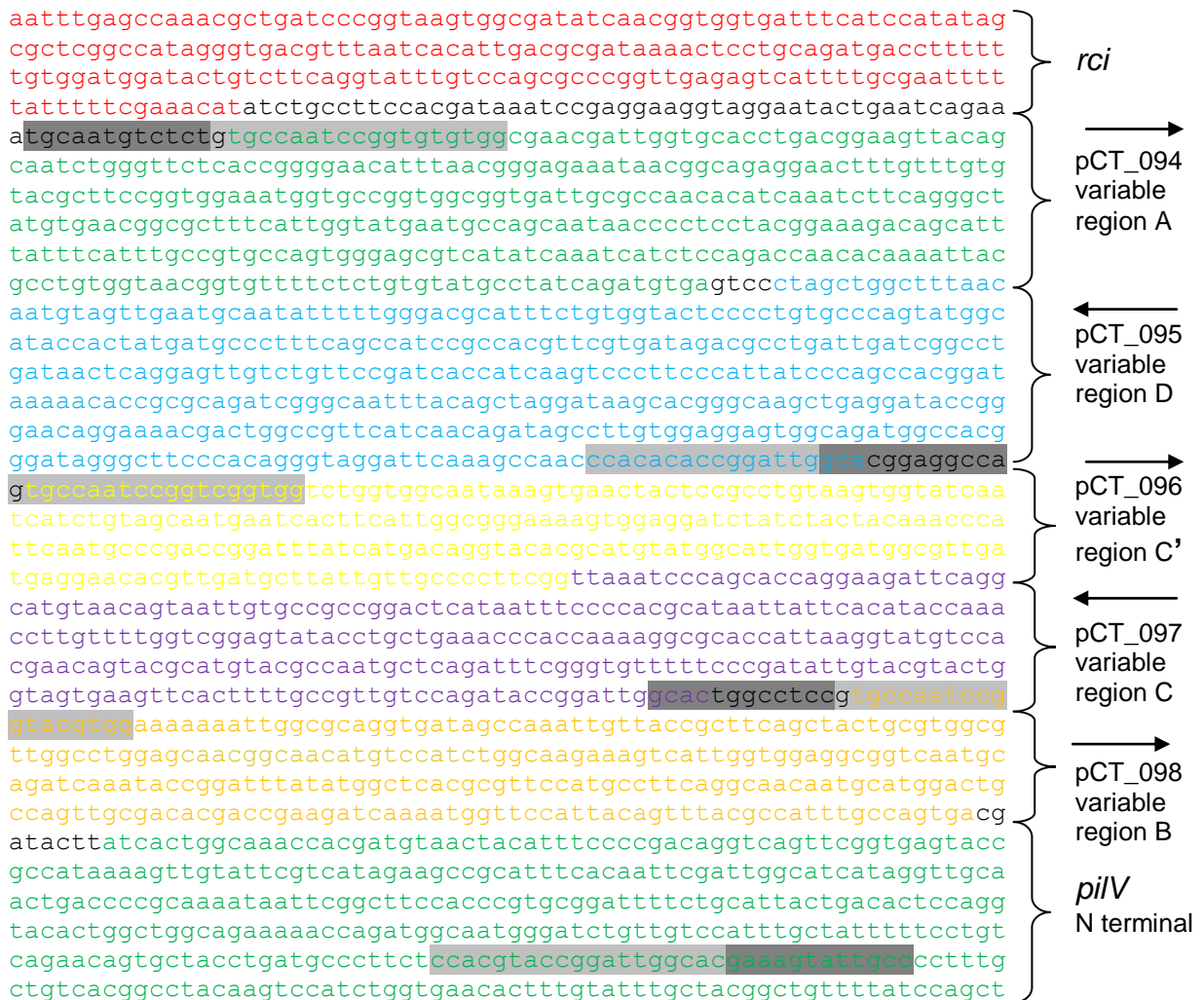
only seen in samples prepared in LB broth. Therefore, it may be that the conditions used in my study favour protrusion production for particular strains, and that if bacteria were grown in an alternative manner a different result might be obtained. Whilst inactivation of either the *pil* or *tra* loci had no phenotypic effects beyond conjugation, the SEM data show both can alter the expression of the 'protrusions', although their precise nature remains to be elucidated.

Associated with the *pil* locus is the shufflon recombinase (*rci*) which facilitates the recombination of *pilV* DNA components, thereby producing alternate protein variants found at the tip of the thin pilus. Different *pilV* shufflon conformations in plasmid R64 determine the affinity of the pilus for LPS molecules expressed on recipient surfaces thereby establishing host specificity (Komano *et al.*, 1995; Komano *et al.*, 2000). Inactivation of *rci* did not affect the conjugation rate or any of the host phenotypes in host strains *E. coli* J53-2 or *E. coli* 3950, with the exception of susceptibility to cefotaxime. The conjugation frequency of pCT4 (*rci::aph*) to recipient *S. Typhimurium* SL1344 rif^R was reduced on both a solid surface and particularly in liquid media. These data suggest that the conformation of the fixed *pilV* shufflon components has a lower affinity for *S. Typhimurium* SL1344 binding receptors than for *E. coli*.

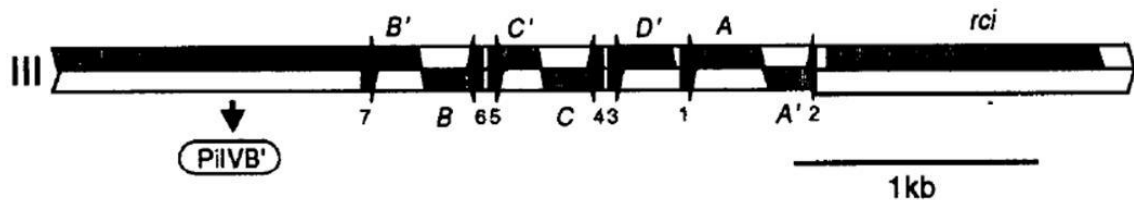
The arrangement of the wild-type pCT shufflon components from the DNA sequence read data had most similarity to the functional shufflon region of IncI plasmid R64 in the B' conformation (Figure 6.19). Komano *et al.*, (1995) found that a fixed R64 shufflon in this conformation had a generally lower frequency of conjugation, but a higher transfer rate into *S. Typhimurium* LT2 than the other fixed conformations (Komano *et al.*, 1995). It is possible that if the DNA sequence assembly was based on the predominant shufflon order encoded within the reads, that the higher than

Figure 6.19. The DNA sequence of the pCT of shufflon region

A



B



A, *rci*, red; pCT_094 variable region A, light green; pCT_095 variable region C', light blue; pCT_096 variable region C', yellow; pCT_097 variable region C, purple; pCT_098 variable region B, orange; pCT_099 *pilV*, dark green; Grey highlighted, identified predicted sfx shufflon binding regions; Light grey, conserved sfx regions.

B, The genetic organisation of R64 shufflon components in the B' conformation Adapted from Komano *et al.*, 1995.

expected pCT conjugation frequency to recipient *S. Typhimurium* SL1344 rif^R in liquid and from *E. coli* donor C159/11 (chapter 4) may be attributed to the predominant production of thin pili with a tip protein encoded by the B' shufflon organisation. The affinity of particular Rci proteins for the different recombination sequences is also thought to determine the abundance of the different thin pilus tip proteins (Tam 2005), therefore it may be that the *rci* gene determines the predominant shufflon organisation and therefore recipient binding and plasmid host range. *S. Typhimurium* SL1344 rif^R pCT4 (*rci::aph*) was significantly less able to invade human tissue culture cells ($P = 0.018$) and was the most attenuated strain in the *C. elegans* infection model. The MIC of cefotaxime required to inhibit all host bacteria containing pCT4 was also consistently a minimum of one doubling dilution higher than bacterial strains containing wild-type pCT. As the same effect was not observed in the *pilS* inactivated plasmid these result cannot be attributed to a lack of pilus binding, however it may be that the absence of the recombinase itself leads to a change in bacterial phenotype when compared to effects conferred by wild-type pCT.

There is homology between the IncI plasmid C shufflon component and *pilV2* of *S. Typhi* SP17 (Komano, 1999; Morris *et al.*, 2003) and further examples in the literature of chromosomally based recombination sites, typically where a promoter is inverted by a recombinase varying expression of downstream genes such as *Salmonella* flagellen genes (H1/H2) or a type I pilus system found in *E. coli* (Freitag *et al.*, 1985; Johnson and Simon, 1985). Many targets for recombination are also genes which encode membrane proteins. Therefore, interaction between plasmid shufflon recombinase and host chromosomal recombination sites may alter binding or entry of

cefotaxime into the cells, accounting for the changes to host phenotype observed in this study (Gyohda *et al.*, 2004).

The last of the pCT genes investigated was the *pndABC* region identified as a putative toxin anti-toxin system and shown in R64 by Furuya and Komano (1996) to contribute to plasmid stability. Inactivation of *pndACB* was the most complex of the five selected genes, as the majority of the kanamycin resistant colonies once visible, became non-viable. The simplest explanation for this phenomenon is that during growth both toxin and anti-toxin RNA were present within the dividing cells. However, as the toxin mRNA remains stable for longer, when the anti-toxin RNA was degraded, toxin was produced and the cells were killed. Those cells found to continue growing may have, by chance, not received a copy of the toxin mRNA upon division. The main problem with this hypothesis is that typically mRNA has a half life of 1-2 minutes. Although *pndACB* anti-toxin mRNA of R483 has been estimated to have a half life of 20 minutes (Nielsen *et al.*, 1991), this is still too short a time to explain the findings as colonies grew for approximately 6-10 hours. It may be that residual wild-type plasmid was present alongside the mutant plasmid over multiple generations due to a high copy number or the formation of plasmid monomers. Cessation of the growth of the majority of colonies suggested that the pCT *pndA* toxin was active, and able to kill the bacterial host cell in the absence of the anti-toxin. Therefore, if a molecule or compound was found that could inhibit the transcription of the *pndB* anti-toxin or prevent binding of *pndB* RNA to the *pndA* toxin mRNA this may provide an effective anti-plasmid (and anti-bacterial) treatment option.

In R64 when *pndACB* was inactivated, ~50% of bacteria lost the plasmid after 60 generations. In a double inactivation not dissimilar to pCT9 (inactivation of *pndACB* and part of the R64 *tra* locus) ~80% of bacteria had lost R64 within this time frame (Furuya and Komano, 1996). The same was not observed when *pndACB* was inactivated in pCT as both pCT8 and pCT9 remained stable over approx 70 generations in all host strains. This was perhaps the most surprising for host *E. coli* J53-2 where it was hypothesised that pCT conferred a fitness burden (chapter 4). Therefore, these data suggest that *pndACB* is not the only gene locus encoding stability and persistence genes on pCT, and that multiple regions are involved in ensuring vertical transfer of this plasmid. Such regions in the pCT genome are yet to be identified.

E. coli DH5α pCT8 and pCT9 (*pndACB::*) remained in a 1:1 ratio when competed against *E. coli* DH5α pCT, however, *E. coli* DH5α pCT9 (*traXY::aph* and *pndACB::cat*) was out-competed when grown with *E. coli* DH5α alone. As pCT7 (*traXY::aph*) was able to compete with the *E. coli* DH5α, this suggests inactivation of both transfer and persistence regions of pCT together had a detrimental effect on the success of pCT. These data add weight to the hypothesis that the fitness of pCT, is in part, due to both successful vertical and horizontal transfer.

Biofilm formation was also investigated as the capacity to form this structure may increase the bacterial strain's ability to survive and persist within diverse and hostile environments as well as enhance resistance to antimicrobials and sterilisation (Hall-Stoodley *et al.*, 2004), thereby increasing bacterial 'fitness' (Pope *et al.*, 2010). Biofilms are also particularly important when considering persistence of *E. coli* and *Salmonella* strains within the farm environment, and their adherence to food

products, as these factors have implications for both animal and human health (Solomon *et al.*, 2005). Despite successfully persisting within the index farm (Liebana *et al.*, 2006), *E. coli* C159/11 (which contains pCT) produced very little biofilm. The addition of pCT and the pCT mutants to the other bacterial host strains also had no effect on the ability of bacterial host strains to form a biofilm. Although several studies have shown that plasmids can contribute to the initial stages of biofilm formation through the production of conjugative pili (Ghigo, 2001; May *et al.*, 2010), this does not appear to be the case for pCT. The increased cell to cell contact within a biofilm also increases plasmid conjugation frequency of some plasmids (Parsek and Singh, 2003). Therefore, an additional experiment to determine whether pCT transfer is affected by the production of host cell biofilm would be of interest.

In conclusion, inactivation of the five selected pCT genes had no universal effect on plasmid persistence, plasmid competitive fitness against wild-type pCT or host strain growth kinetics and the ability of all the host bacterial strains tested to cause infection. Inactivation of the pili genes *traXY* prevented pCT conjugative transfer, and inactivation of *pilS* reduced the frequency of pCT conjugation to recipient *S. Typhimurium* SL1344 rif^R particularly in liquid, potentially revealing a role for the thin pilus in pCT transfer to selected hosts. Inactivation of these pCT pilus genes also reduced the number of *E. coli* DH5 α and *S. Typhimurium* cells which produced protrusions, visualised using SEM.

Natural pCT host strain, *E. coli* C159/11 was not able to form a biofilm, and the addition of wild-type pCT and the pCT mutants also had no effect the ability of all host strains to produce a biofilm or to aggregate together in any of the conditions tested in my study.

6.9 Further work

A targeted approach to inactivating pCT genes was taken as the sequence analysis and previous literature suggested that the seven genes selected (*bla*_{CTX-M-14}, *ISEcp1*, a putative sigma factor gene, *rci*, *pilS*, *traXY* and *pndACB*) may be associated with plasmid persistence and success. An alternative method which could be considered for future work is the systematic disruption of all the pCT CDSs using TraDIS (transposons-directed insertion site sequencing). TraDIS uses the insertion of a Tn5 derivative with outwardly orientated promoters to allow subsequent rapid identification of the disrupted genomic region using DNA sequencing (Langridge *et al.*, 2009). Conventionally this method has been used to identify essential chromosomal genes for bacterial growth and survival (*in vitro* and *in vivo*) or genes required when the bacterial cell is placed under certain conditions e.g. in the presence of bile (Langridge *et al.*, 2009; Eckert *et al.*, 2011). Phan (2009) used TraDIS to identify genes on a *S. Typhi* plasmid pHCM1 (IncHII) and its bacterial host, responsible for stable maintenance of this plasmid. Serial passage (~60 generations) of the estimated 1.1×10^9 mutant strains revealed those which lost the plasmids, therefore identifying the mutations which perturbed the stability of pHCM1 (Phan, 2009). A similar approach using TraDIS to create a pCT mutant library could be used to investigate which genes are required for pCT stability or genes involved in conjugation by measuring the ability of each mutant to conjugate. Genes required for the persistence and dissemination of pCT *in vivo* could also be identified in a similar approach to that of Eckert *et al.*, (2011) for *E. coli* 0157:H7, by administering bacteria containing the pCT mutant library to *C. elegans*, cattle or mice and monitoring which strains are less able to survive in an *in vivo* environment.

A global overview of pCT and bacterial host gene expression in differing conditions is also a desirable future experiment. The most appropriate technology currently available to do so is Whole Transcriptome Shotgun Sequencing (WTSS) or RNA-seq, which allows the characterisation of the bacterial transcriptome under various different conditions using sequencing of mRNA (Wang *et al.*, 2009; Pinto *et al.*, 2011). Although RNA-seq has almost exclusively been used to identify mRNA of chromosomal genes, Isabella and Clark (2011) showed that plasmid pJD1 from *Neisseria gonorrhoeae* differentially expressed *repA* (replication gene), *vapDX* (toxin antitoxin operon), and *mobAB* (mobilisation genes) when the host strain was grown in aerobic/anaerobic conditions (Isabella and Clark, 2011). Therefore, this technology could be used initially to determine changes in the bacterial host gene expression when pCT is present or absent, to determine the pCT CDSs which are functional and therefore expressed, and to measure changes in pCT gene expression within different bacterial host strains or when host strains are grown in different environments. Investigation of the pCT transcriptome will also show whether the pCT putative sigma factor is expressed, and if so under what conditions, informing on the possible role of this unusual plasmid gene.

Other future work could consider anti-plasmid treatment options (Section 1.4.5), by screening for molecules which are able to prevent the transcription or bind to anti-toxin component *pndB*. Such molecules should be easier to identify than curing agents as successful inhibition of the antitoxin will result in cell death, detectable both in culture and using flow cytometry with a viable cell dye.

The confirmed production and clear importance of the pCT conjugation apparatus provides an opportunity for targeted blocking of either one or both of the pCT pili

using bacteriophages. This could potentially provide a feasible anti-plasmid prophylactic or therapeutic treatment to prevent pCT dissemination. Although many phage have been identified that absorb to either the tip or the shaft of pili produced by IncI type plasmids (Hedges and Datta, 1973; Coetzee *et al.*, 1980), phages I α , If1, I $_2$ -2 and PR64F8 are all unable to absorb to pili produced by IncK and IncB plasmids (Coetzee *et al.*, 1982). Therefore screening for a phage able to absorb to the pCT thick or thin pilus and reduce conjugation could be undertaken, perhaps by collection of samples from diverse environments such as soils. The outer protein coat of any candidate phage should also be investigated for an ability to bind to pCT pili, as crude phage protein preparation have been shown to have anti-conjugation action, and would be extremely desirable as a treatment option (Lin *et al.*, 2011). Phage which absorb to, and are internalised by pCT pili could also be used to deliver other potential anti-plasmid DNA or RNA to bacterial host cells, for example to bind to the antitoxin RNA of the PSK system *pndACB* allowing production of the toxin PndA and subsequent killing of the bacterial cell. In addition, if the *S. Typhimurium* LPS receptor for the pCT *pilV* epitope of the thin pilus was identified, blocking this receptor may prevent the efficient transfer of pCT to pathogenic *S. Typhimurium* strains and act as a anti-salmonella strategy at specific outbreak sites.

6.10 Key findings

- The pCT *pndACB* region is not the exclusive factor which allows pCT to persist within host cells.
- Inactivation of the pCT putative sigma factor had little effect on host fitness and plasmid success.
- The *tra* locus is essential for pCT transfer by conjugation.

- The active *pil* locus is not required for pCT liquid conjugation although it does increase the transfer of frequency in liquid and on a solid surface to recipient *S. Typhimurium* SL1344 *rif*^R.
- The residence of pCT and corresponding *pil* locus did not affect the host bacterium's ability to form a biofilm, to aggregate or to adhere to eukaryotic cells
- Addition of wild-type pCT modified the appearance of *E. coli* DH5α and *S. Typhimurium* SL1344 *rif*^R cells when visualised by scanning electron microscopy. The protrusions seen may be linked to *tra* and *pil* pilus production.

Chapter 7:

**Overall
Discussion**

7. Overall Discussion

The ability of plasmids to acquire and disseminate genetic material has facilitated the rapid horizontal transfer of clinically relevant antibiotic resistance and virulence genes to new strains, and between bacterial species. Plasmids are also instrumental in the accumulation of antibiotic resistance determinants, resulting in multi-drug resistant (MDR) bacteria which pose a threat to the continued treatment of infections using antibiotics in the future (Maltezou, 2009; Bush and Fisher, 2011). While the use of antimicrobials both clinically and in agriculture has undoubtedly led to the increased prevalence of antibiotic resistant bacterial strains and genes (Davies and Davies, 2010; Dhanji *et al.*, 2010; Walsh *et al.*, 2011), a better understanding of the mechanisms by which conjugative plasmid backbones are able to persist and disseminate within bacterial populations will aid in efforts to manage and treat antibiotic resistant bacterial infections and delay the surge of pan-resistant bacterial strains.

In my study *bla*_{CTX-M-14} carrying plasmid, pCT, was chosen for detailed investigation. Previous data had shown that pCT conferred resistance to third generation cephalosporins and successfully persisted and spread within livestock and the environment on a dairy farm over a three year period (Liebana *et al.*, 2006). pCT was also present in at least six different *E. coli* host strains on the index farm, indicating extensive plasmid transfer rather than a clonal expansion of a particular host strain, making pCT a suitable candidate for further study of factors influencing plasmid success.

Complete DNA sequencing and annotation revealed that pCT was 93,629 bp in size and encoded all three IncI group associated regions, the *repYZ*, *tra* and *pil* loci.

Comparison of the pCT genome to other *bla*_{CTX-M} encoding plasmids showed no conserved regions with the exception of the antibiotic resistance gene itself. Therefore, it seems no one common genomic factor enabled the acquisition or maintenance of *bla*_{CTX-M} genes on these conjugative plasmids, supporting the hypothesis of multiple acquisitions of *bla*_{CTX-M} genes by various different plasmid backbones. Further comparison of the sequence of pCT with other, similar plasmid sequences deposited in genbank allowed the identification of conserved DNA regions found in similar IncI and IncK reference plasmids R64 and R387, respectively, but also novel and unique regions when compared to the four plasmids with the highest degree of homology to pCT (pO26_vir, pR3521, pO113 and pSERB1). The detailed analysis of the pCT genome facilitated the development of specific PCR assays designed to amplify a selection of pCT genes situated around the genome. This test provided a rapid and high-throughput pCT detection test which was able to discriminate the presence of pCT from other plasmids to a much greater degree than currently used Inc typing methods. The analysis of plasmid *nikB* sequences also proved to be a very useful tool to identify homology and relationships between IncI plasmids. In my study use of the PCR assay identified ten pCT-like plasmids in UK veterinary isolates and human clinical isolates from China, Australia and Spain, including plasmid pRYC105 which is distributed throughout Spain in both community acquired infections and in the environment (Valverde *et al.*, 2009). A subsequent study by the Animal Health and Veterinary Laboratories Agency using the same PCR tests showed a further twenty-one pCT-like plasmids from UK cattle and turkey farms and clinical isolates from Wales (Stokes *et al.*, Submitted). These data together reveal that plasmid pCT is widespread globally, and is a common vector for

dissemination of *bla*_{CTX-M-14} in bacteria isolated from humans, animals and the environment. They also show direct evidence for the movement of this mobile genetic element between bacteria within these different niches.

The pCT genome analysis also showed *bla*_{CTX-M-14} to be the only antibiotic resistance gene encoded on the plasmid; therefore persistence and spread of pCT could not be attributed to co-selection associated with pressure from non β -lactam antimicrobial drugs. This finding suggested that either pCT containing bacteria had been constantly exposed to β -lactam antibiotic compounds, which due to its widespread distribution in many different environments seems unlikely; or that pCT is able to remain stably persist and disseminate in the absence of antibiotic selective pressure. To investigate this hypothesis the stability of plasmid pCT and any fitness cost or benefit the plasmid conferred upon bacterial host strains 'pCT naive' *E. coli* DH5 α , *E. coli* J53-2 and *S. Typhimurium* SL1344 rif^R, and the pCT 'cured' C159/11 derivative, *E. coli* 3950, were assessed.

pCT was stably maintained in the absence of β -lactam antibiotic pressure, in the four bacterial host strains over approximately 70 generations. Similarly, each pCT mutant (pCT2-9) remained stable over this time period indicating that the genes selectively inactivated (*bla*_{CTX-M-14}, putative sigma factor, *rci*, *pilS*, *ISEcp1*, *traXY* and *pndACB*) were not individually responsible for the short term maintenance of pCT. This was perhaps the most surprising in the case of pCT8 and pCT9 which had an inactivated putative toxin-antitoxin system *pndACB*, which appeared to be active during the construction of these plasmid mutants. When a homologous *pndACB* operon was inactivated on plasmid R64, this significantly reduced the stability of the plasmid, so much so that after approx 100 generation the majority of bacterial cells were R64 free

(Furuya and Komano, 1996). Therefore, my data infers that pCT either encodes another currently unknown addiction or partitioning system which possibly works together with *pndACB*, or, other mechanisms such as a low fitness burden conferred upon the host, or high pCT copy number allow maintenance of this plasmid in all daughter bacterial cells without addiction.

To investigate the fitness burden/benefit of pCT, growth kinetics were measured for each bacterial host strain +/- pCT. The addition of pCT had no significant effect on the generation time of the four host strains grown isogenically and pCT7 (non-conjugative pCT mutant) appeared to confer neither a competitive advantage or disadvantage to *E. coli* host DH5 α cells when grown in competition with the plasmid free parental strain. These results suggested that pCT does not confer a fitness cost or benefit to the pCT 'naive' bacterial host strain *E. coli* DH5 α despite the lack of a period of co-evolution between bacterial host and plasmid. Therefore, the stability of pCT and absence of a fitness burden on host bacteria strains *E. coli* DH5 α (and *E. coli* 3950) predicts that in the absence of antibiotic pressure, pCT will persist once established in bacterial populations regardless of antibiotic administration or stewardship; and that amplification will occur if exposed to selective antibiotics. Although pCT remained stable and did not affect the generation times of the third host strain, *E. coli* J53-2, competition assays revealed that wild-type pCT conferred a fitness burden on this bacterial host. However, further investigation showed that pCT2 conferred less of a fitness cost on *E. coli* J53-2 than wild-type pCT, inferring that at least part of the pCT burden was caused by the presence of the *bla*_{CTX-M-14} gene.

Other competition data showed that *E. coli* DH5 α containing the pCT mutant plasmids pCT3 - 9 remained in a 1:1 ratio when competed against *E. coli* DH5 α containing wild-type pCT, indicating the inactivation of the selected pCT genes neither increased nor decreased the competitive fitness of the *E. coli* DH5 α host strain. The addition of pCT also had no significant effect on bacterial host pathogenicity, on the ability of bacterial host strains to form a biofilm, or to aggregate. Therefore, with the exception of resistance to β -lactams and the extracellular protrusions observed using scanning electron microscopy, the acquisition of pCT conferred no major changes to the host strain phenotypes.

The conjugation frequency of pCT was measured from two donor *E. coli* strains to several recipient strains (*E. coli* and *S. Typhimurium*) in a liquid and on a solid surface. Comparative transfer rates of pili inactivated mutant plasmids pCT5 (*pilS::aph*) and pCT7 (*traXY::aph*) were also assessed to establish the role of these pili in the conditions tested. Data collected indicated that transfer to host recipient *E. coli* J53-2 was exclusively by the thick pilus and conjugation to *E. coli* J53-2 was at a high rate, particularly on a solid surface. pCT conjugation in liquid was more frequent than on a solid surface to recipients *E. coli* 3950 (derived from *E. coli* C159/11) and *S. Typhimurium* SL1344 rif^R, and involved conjugation by both the thick and thin pili. Inactivation of the shufflon recombinase (pCT4) and *pilS* (pCT5), preventing conformational changes at the tip of the thin pilus and pilus production respectively, had a detrimental effect on pCT conjugation frequency to recipient *S. Typhimurium*. Therefore, it seems that the *pil* locus and conjugation in liquid is most important for the transfer of pCT to recipient strain *S. Typhimurium* SL1344 rif^R, and that the pCT *pil* locus may play a role in expanding the host range of this plasmid. The ease with

which pCT was able to conjugate to *S. Typhimurium* SL1344 rif^R in my experiments may have clinical or veterinary implications as the acquisition of antibiotic resistance genes (and virulence genes on other similar plasmids) by pathogenic *Salmonella enterica* strains may lead to significant treatment failures. Within the farm environment, *E. coli* and *Salmonella* strains are very often co-located (Poppe *et al.*, 2005) and may be exposed to low level β -lactams such as ceftiofur (Daniels *et al.*, 2009), potentially selecting for the spread of pCT between these two species. As screening for pCT-like plasmids in *S. enterica* strains was beyond the scope of this project the prevalence of pCT in this species is currently unknown. The importance of the *pil* locus in pCT transfer to *S. Typhimurium* lends itself to possible treatment options which target this pilus, potentially useful in an outbreak situation to prevent further spread of pCT-like plasmids to more pathogenic bacterium.

The generation of a novel method to specifically and rapidly inactivate targeted plasmid genes allowed analysis of the consequences arising from the disruption of seven pCT gene regions. While inactivation of these genes had little effect on the host phenotypes investigated within my study, development of the method will allow examination of other genes of interest both on pCT (for example the annotated hypothetical proteins) or genes on other large conjugative plasmids in a similar way, opening up a functional genomics approach which will be useful as many more plasmid genomes are sequenced.

A common theme during this project was bacterial host specific differences seen throughout all my experiments. I found that the bacterial host strain determined the conjugation frequency and the fitness cost of both plasmid and antibiotic resistance gene. Therefore the bacterial host strain influenced the success of pCT. Bacterial

strain specific differences have probably been underestimated in previous studies of plasmids, especially when using one particular strain (typically *E. coli* J53 or *E. coli* DH5 α /K12) and extrapolating the findings to other host strains or other plasmids. Therefore, continued study of plasmids must take into account host diversity. Investigation to determine the bacterial host or plasmid factors which create these differences is also an interesting avenue for further work. While my project examined just one conjugative plasmid, within four different bacterial hosts, my data alludes to the extensive variation and complex nature of the relationship between plasmid and bacterial host. It may be that particular host strain and plasmid backbone partnerships are better suited to different environment conditions. For example, a notable host and plasmid pairing is epidemic strain *E. coli* O25:H4-ST131 and IncF plasmids (often encoding *bla*_{CTX-M-15}) found in both human and veterinary samples (Ewers *et al.*, 2010; Xu *et al.*, 2011). *E. coli* ST131 is a pathogenic strain which typically carries numerous antibiotic resistance genes (both chromosomally and plasmid encoded), causing major treatment issues and often leading to the use of last resort antibiotics, carbapenems (Woodford *et al.*, 2009; Peirano and Pitout, 2010). It may be that this type of multidrug resistant strain/plasmid combination is better adapted to environments where antibiotic selective pressures are numerous.

Conversely, pCT-like plasmids have been shown to be present in >10 different *E. coli* clonal types (Liebana *et al.*, 2006; Coldham *et al.*, 2011) and have no association with one particular bacterial strain. Host strains containing pCT-like plasmids have largely been isolated from community acquired urinary tract and infections, as commensals of healthy patients and in the farm environment (Valverde *et al.*, 2004; Liebana *et al.*, 2006; Valverde *et al.*, 2009) where a constant antibiotic presence is

unlikely. Therefore, it seems that pCT is a promiscuous plasmid, able to conjugate and persist within many different *Enterobacteriaceae* host strains without a constant antibiotic presence. This may be in part due to the apparent lack of a fitness cost conferred to even naive bacterial hosts (*E. coli* DH5 α), which in combination with the postulated addiction systems, may allow the rapid establishment and vertical transmission of pCT. These factors contribute to the hypothesis that pCT has adapted to persist in the absence of antibiotic pressures, probably prior to the acquisition of the *bla*_{CTX-M-14} gene and the use of third generation cephalosporins, and that stable incorporation of this single antibiotic resistance gene has had little effect on plasmid function or the niche that it occupies. This hypothesis corresponds with recent work by Iredell *et al.* (2011) who showed that plasmids which did not encode antibiotic resistance genes were common within samples and had similar backbones to many plasmids which do carry common antibiotic resistance genes (Iredell *et al.*, 2011).

The high frequency of pCT transfer in liquid, the importance of the *pil* locus, and lack of *E. coli* C159/11 biofilm production may also suggest that plasmid pCT (and host) have adapted to a liquid environment. Rather than surviving within a static biofilm, rapid liquid conjugation to a number of different hosts may allow better spread and survival of the plasmid itself. It is conceivable that the combination of efficient dissemination of pCT to a variety of bacterial host strains, followed by successful stability within these bacteria due to a low fitness cost and the hypothesised presence of multiple addiction systems has aided this plasmid in its worldwide dissemination and persistence.

Data and hypotheses collated within my project strongly indicate that simple cessation or limitation of the use of antibiotics through antibiotic stewardship is unlikely to remove the antibiotic resistance gene vectors from bacterial populations, leading to the amplification of these plasmids when use of the antibiotic is recommenced. Therefore, the control of plasmids such as pCT, by limiting the dissemination and prevalence of these plasmids may be a more realistic therapeutic strategy, particularly in hospitals and agricultural environments. This could be achieved by the identification of anti-plasmid curing agents, compounds which allow the production of toxins encoded within the toxin-antitoxin systems (such as *pndACB*), leading to cell death, or by prevention of conjugation by targeting the pili apparatus or relaxase mechanisms. These measures would lower the risk of plasmid transmission to a) more clinically relevant pathogens such as toxigenic *E. coli* strains or *S. enterica* b) to bacteria already containing plasmids encoding virulence or other antibiotic resistance genes with which pCT could recombine (e.g. pO26_vir), or c) multi-drug resistant bacterial strains.

In conclusion, plasmid pCT was shown to be widely distributed in *E. coli* isolated from humans and animals. Success of this plasmid is postulated to be due to a combination of subtle factors rather than one particular gene or phenotypic benefit conferred to host strains. These factors include stability within a range of hosts, a lack of a fitness burden conferred to new host strains allowing establishment of the plasmid, and the proficient conjugation of pCT to a range of bacterial hosts in both liquid and on solid media. There is still much to learn about the complex nature of plasmid and bacterial host strain interactions with regard to plasmid functions such as conjugation and stability, plasmid and bacterial host gene expression and how

different phenotypes affect the overall evolutionary fitness of plasmid and host in different conditions. Therefore, the hypotheses and methods developed during my study using plasmid pCT provide a resource for further investigation.

Publications resulting from this study

Original Articles

Jennifer L. Cottell, Mark A. Webber, Nick G. Coldham, Dafydd L. Taylor, Anna M. Cerdeño-Tárraga, Heidi Hauser, Nicholas R. Thomson, Martin J. Woodward and Laura J.V. Piddock, (2011) Complete Sequence and Molecular Epidemiology of an IncK Epidemic Plasmid Encoding *bla*_{CTX-M-14} Widely Disseminated in Humans and Animals. **Emerging Infectious diseases**; **17**, Issue 4, 645-652.

Jennifer L. Cottell, Mark A. Webber and Laura J.V. Piddock. Persistence of transmissible antibiotic resistance in the absence of antibiotic pressure. **Submitted to Lancet Infectious diseases, 2011**

Matthew Stokes, **Jennifer L Cottell**, Laura J.V.Piddock, Guanghui Wu, Mandy Wootton, Luke Randall, Chris Teale, Mark Fielder and Nick Coldham. Dissemination of *bla*_{CTX-M-14} by an IncK plasmid vector in *E. coli* isolates from humans, turkeys and cattle in England and Wales. **Submitted to Emerging Infectious diseases, 2011**

Conference Presentation

J.L. Cottell, M.A. Webber and L.J.V. Piddock (2011) A new method to inactivate genes on large conjugative plasmids for investigation of their biological function. **European Congress of Clinical Microbiology and Infectious diseases (ECCMID)** 7- 10th May 2011, Milan, Italy.

J.L. Cottell, M.A. Webber, D.L. Taylor, N.G. Coldham, N.R. Thomson, M.J. Woodward and L.J.V. Piddock (2010) Complete Sequence and Epidemiology of a Widely Disseminated IncK Epidemic Plasmid Encoding *bla*_{CTX-M-14}. **Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC)** 12-15th September 2010, Boston USA.

Coldham N.G., Stokes M., **Cottell J.**, Randall L., Wearing H., Snow L., Wootton M., Howe R., Piddock L.J., Teale C. (2011) Molecular epidemiology of CTX-M14 ESBL *E.coli* from cattle and humans in England and Wales, **European Congress of Clinical Microbiology and Infectious diseases (ECCMID)** 7- 10th May 2011, Milan, Italy.

Invited oral presentation "Characterisation of a *bla*_{CTX-M-14} carrying epidemic plasmid pCT"
Jennifer L Cottell. British Society of Antimicrobial Chemotherapy, Antimicrobial Research Mechanisms Workshop, November 2010.

Appendix

Appendix 1. Sequence of CTX-M Group 9 PCR amplicon derived from pCT aligned with known *bla*_{CTX-M-14} sequence

| | |
|--|--|
| <i>bla</i> _{CTX-M-14} pCT sequence | gatgatgttc gcggcggcgg cgtgcattcc gctgctgctg ggcagcgcgc cgctttatgc gcagacgagt gcggtgcagc CGGATATGTT GCGGCGGCGG CGTGCATTCC GCTGCTGCTG GGAAGAGCGC CGCTTTATGC GCATACGAGT GCGGTGCAGC |
| <i>bla</i> _{CTX-M-14} pCT sequence | aaaagctggc ggcgctggag aaaagcagcg gagggcggct gggcgtcgcg ctcatcgata ccgcagataa tacgcaggtg AAAAGCTGGC GGCCTGGAG AAAAGCAGCG GAGGGCGGCT GGGCGTCGCG CTCATCGATA CCGCAGATAA TACGCAGGTG |
| <i>bla</i> _{CTX-M-14} pCT sequence | ctttatcgcg gtgatgaacg ctttccaatg tgcagtagca gtaaagttat ggcgcccgcg gcggtgctta agcagagtga CTTTATCGCG GTGATGAACG CTTTCCAATG TGCAGTAGCA GTAAAGTTAT GGCGCCGCG GCGGTGCTTA AGCAGAGTGA |
| <i>bla</i> _{CTX-M-14} pCT sequence | aacgcaaaag cagctgctta atcagcctgt cgagatcaag cctgccgacg tggttaacta caatccgatt gccgaaaaac AACGCAAAAG CAGCTGCTTA ATCAGCCTGT CGAGATCAAG CCTGCCGATC TGGTTAACTA CAATCCGATT GCCGAAAAAC |
| <i>bla</i> _{CTX-M-14} pCT sequence | acgtcaacgg cacaatgacg ctggcagaaac tgagcgcggc cgcggttcag tacagcgaca ataccgccat gaacaaattg ACGTCAACGG CACAATGACG CTGGCAGAAC TGAGCGCGGC CGCGTTGCAG TACAGCGACA ATACCGCCAT GAACAAATTG |
| <i>bla</i> _{CTX-M-14} pCT sequence | attgcccagc tcggtggccc gggaggcgtg acggcctttt cccgcgcgat cggcgatgag acgtttcgctc tggatcgcac ATTGCCCAGC TCGGTGGCCC GGGAGGCGTG ACGGCTTTTG CCCGC CGCAT CCGCGATGAG ACGTTTCGTC TGGATCGCAC |
| <i>bla</i> _{CTX-M-14} pCT sequence | tgaacctacg ctgaataaccg ccattcccgg cgacccgaga gacaccacca cgccgcgggc gatggcgagc acgttgcgtc TGAACCTACG CTGAATAACG CCATTCCCGG CGACCCGAGA GACACCACCA CGCCGCGGGC GATGGCGCAG ACGTTGCGTC |
| <i>bla</i> _{CTX-M-14} pCT sequence | agcttacgct ggggtcatgag ctgggcgaaa cccagcgggc gcagttgggtg acgtgggtca aaggcaatac gaccggcgca AGCTTACGCT GGGTCATGCG CTGGGCGAAA CCCAGCGGGC GCAGTTGGTG ACGTGGCTCA AAGGCAATAC GACCGGCGCA |
| <i>bla</i> _{CTX-M-14} pCT sequence | gccagcattc gggccggcctt accgacgtcg tggactgtgg gtgataagac cggcagcggc gactacggca ccaccaatga GCCAGCATTC GGGCCGGCTT ACCGACGTCG TGGACTGTGG GTGATAAGAC CGGCAGCGGC GACTACGGCA CCACCAATGA |
| <i>bla</i> _{CTX-M-14} pCT sequence | tattgcggtg atctggccgc agggctcgtgc gccgctgggt ctggtgacct attttaccca gccgcaacag aacgcagaga TATTGCGGTG ATCTGGCCGC A?GGTCGTGC GCCGCTGGTT CTGGTGACCT ATTTTACCCA GCCGCAACAG AACGCAGAGA |
| <i>bla</i> _{CTX-M-14} pCT sequence | gccgcccgca tgtgctgg GCCGCCGCGA TGTGCTGG |

Amplicons sequenced from CTX-M Group 9 PCR were homologous to known *bla*_{CTX-M-14} nucleotide sequences accessed from genbank accession no. AF252622 highlighted in grey.

Appendix 2. pCT-like plasmid *E. coli* DH5 α transformants

| Lab code | Strain | Plasmid | Inc type |
|-----------------|----------------------|----------------|-----------------|
| I779 | DH5 α pI779 | pI779 | K/F |
| I780 | DH5 α pI780 | pI780 | K/F |
| I801 | DH5 α pC559 | pC559 | K |
| I802 | DH5 α pC567 | pC567 | K |
| I803 | DH5 α pC574 | pC574 | K |
| I853 | DH5 α pRYC105 | pRYC105 | K |
| I832 | DH5 α pOZ174 | pOZ174 | - |
| I849 | DH5 α pJIE052 | pJIE052 | B |
| I850 | DH5 α pJIE201 | pJIE201 | K |
| I865 | DH5 α pJIE182 | pJIE182 | B |

Appendix 3. *C. elegans* culture media

Nemotode Growth Media (NGM)

Make up to 1 litre in SDW

- 17 g of bacteriological agar
- 2.5 g of peptone
- 2.5 g of peptone
- 1ml of cholesterol* (5 mg/ml) in EtOH

Autoclave, cool to 50⁰C then add:

- 25 ml of PBS (pH6)
- 1ml of CaCl (1 M)
- 1ml of MgSO₄ (1 M)

M9 Buffer

Made up to 1 litre in SDW

- 3 g of KH₂PO₄
- 6 g of Na₂HPO₄
- 5 g NaCl

Autoclave, cool to 50⁰C and add:

- 1ml of MgSO₄ (1 M)
- 1ml of CaCl₂ (1 M)

*Cholesterol, Sigma Aldrich, UK, Cat. no. EC 200-353-2

Appendix 4. Generation times of host strains +/- pCT

| Lab code | Strain | Generation time (mins) | SD | Student T-test (<i>P</i>) |
|----------|---|------------------------|-------|-----------------------------|
| L1078 | <i>S. Typhimurium</i> SL1344 rif ^R | 88.21 | 6.00 | |
| L1079 | <i>S. Typhimurium</i> SL1344 rif ^R pCT | 94.15 | 11.70 | 0.45 |
| I823 | <i>E. coli</i> 3950 | 64.72 | 2.06 | |
| I834 | <i>E. coli</i> 3950 pCT | 67.94 | 4.52 | 0.28 |
| I825 | <i>E. coli</i> DH5α | 92.61 | 6.70 | |
| I755 | <i>E. coli</i> DH5α pCT | 94.15 | 9.06 | 0.94 |
| I847 | <i>E. coli</i> J53-2 | 63.45 | 4.70 | |
| I855 | <i>E. coli</i> J53-2 pCT | 67.81 | 3.75 | 0.18 |

Growth of each bacterial host strain +/- pCT was measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Figure 4.1, page 142).

Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are represented with bold text.

Appendix 5. The proportion of pCT carrying strains within a population

| <i>E. coli</i> pairing | Percentage of bacteria containing pCT | |
|-----------------------------------|---------------------------------------|----------------------------|
| | 12h (before passage) | 72h (after three passages) |
| A 3950 + 3950 pCT | 63% | 100% |
| B DH5 α + DH5 α pCT | 51% | 84% |
| C J53-2 + J53-2 pCT | 46% | 12% |

The percentage of plasmid pCT carrying bacteria when *E. coli* strains +/- pCT were co-cultures in a 1:1 ratio *in vitro* (Figure 4.2, page 143)

Appendix 6. The ability of *S. Typhimurium* SL1344, SL1344 rif^R and SL1344 rif^R containing pCT to adhere to and invade human intestinal cells

| | SL1344 rif^R (L1078) | SL1344 (L354) | SL1344 rif^R pCT (L1079) |
|--------------------|--|--------------------------|--|
| A: Adhesion | 100% | 307% (<i>P</i> = 0.10) | 93% (<i>P</i> = 0.93) |
| B: Invasion | 100% | 1300% (<i>P</i> = 0.11) | 168% (<i>P</i> = 0.23) |

The number of *S. Typhimurium* SL1344 and SL1344 rif^R pCT bacterium to adhere to (A) and invade (B) INT-407 human intestinal cell is represented as a percentage of the number of *S. Typhimurium* SL1344 rif^R able to do so under test conditions (Figure 4.4, p147). Data are displayed as a mean of three separate experiments all performed with four technical repeats.

Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are represented with bold text.

Appendix 7. The ability of bacterial hosts +/- pCT to cause infection in *C. elegans*

| Lab number | Strain | TD ₅₀ (Days) | Chi ² between curves | P value | Significance (<0.05) |
|------------|-----------------------------|-------------------------|---------------------------------|---------------|----------------------|
| L1078 | SL1344 rif ^R | 3.5 | | | |
| L1079 | SL1344 rif ^R pCT | 3.15 | 3.385794 | 0.0658 | Not significant |
| L354 | SL1344 | 3.9 | 4.87133 | 0.0154 | Significant |
| I823 | 3950 | 4.75 | | | |
| I834 | 3950 pCT | 4.75 | 0.0186159 | 0.8915 | Not significant |

Survival curves were constructed using a Kaplan Mier calculation showing the probability of *C. elegans* survival each day when fed continuously on the tested bacterial strains (Figure 4.5, page 148).

TD₅₀ = time taken for 50% of the nematodes to die,

A significance difference in killing rate was defined as a P value <0.05 denoted by bold text.

Appendix 8. pCT conjugation frequency time optimisation on solid media

| pCT donor strain | Recipient strain | Conjugation frequency / donor cell | | | |
|------------------------------|---|------------------------------------|-----------------------|-----------------------|-----------------------|
| | | 2 hours | 3 hours | 4 hours | 6 hours |
| <i>E. coli</i> C159/11 (pCT) | <i>E. coli</i> DH5α | 4.19×10^{-5} | 5.60×10^{-3} | 8.80×10^{-4} | 6.80×10^{-2} |
| <i>E. coli</i> DH5α pCT | <i>S. Typhimurium</i> SL1344 rif ^R | 8.5×10^{-8} | 7.30×10^{-7} | 1.30×10^{-6} | 6.67×10^{-7} |
| <i>E. coli</i> C159/11 (pCT) | <i>S. Typhimurium</i> SL1344 rif ^R | 2.00×10^{-6} | 4.10×10^{-6} | 1.10×10^{-5} | 1.26×10^{-5} |

The pCT conjugation frequencies were measured from donor strains *E. coli* C159/11 and *E. coli* DH5α during a 2,3,4 and 6 hour period to optimise the the timing for future conjugation experiments (Figure 4.6, page 150) on a minimum of three separate occasions.

Appendix 9. Conjugation frequencies of pCT on solid and in liquid media

A, Conjugation from donor *E. coli* DH5 α (pCT) to various recipients

| Recipient | Media | Average conjugation frequency | Standard deviation |
|---|--------|-------------------------------|-----------------------|
| <i>E. coli</i> J53-2 (rifampicin ^R) | Filter | 1.70×10^{-2} | 1.65×10^{-2} |
| | Liquid | 9.10×10^{-5} | 6.06×10^{-5} |
| <i>E. coli</i> DH5 α (rifampicin ^R) | Filter | 9.65×10^{-5} | 4.53×10^{-5} |
| | Liquid | 2.90×10^{-4} | 9.76×10^{-5} |
| <i>E. coli</i> 3950 (tetracycline ^R) | Filter | 6.12×10^{-6} | 1.27×10^{-6} |
| | Liquid | 1.39×10^{-4} | 1.52×10^{-4} |
| S. Typhimurium SL1344 rif ^R (rifampicin ^R) | Filter | 1.07×10^{-5} | 6.92×10^{-6} |
| | Liquid | 9.33×10^{-6} | 7.69×10^{-6} |

(Figure 4.7, page 152)

B, Conjugation from donor *E. coli* C159/11 (pCT) to various recipients

| Recipient | Media | Average conjugation frequency | Standard deviation |
|---|--------|-------------------------------|-----------------------|
| <i>E. coli</i> J53-2 (rifampicin ^R) | Filter | 1.51×10^{-3} | 1.37×10^{-3} |
| | Liquid | 9.59×10^{-7} | 4.50×10^{-7} |
| <i>E. coli</i> DH5 α (rifampicin ^R) | Filter | 1.56×10^{-6} | 8.66×10^{-7} |
| | Liquid | 9.31×10^{-8} | 9.70×10^{-8} |
| S. Typhimurium SL1344 rif ^R (rifampicin ^R) | Filter | 3.58×10^{-5} | 1.20×10^{-5} |
| | Liquid | 1.63×10^{-4} | 1.30×10^{-4} |

(Figure 4.8, page 153)

Conjugation frequencies were calculated from the mean of at least three separate experiments for each mating pair on a solid surface (filter) and in liquid media.

Appendix 10. Conjugation frequencies on solid media of pCT in the presence of sub-inhibitory concentrations of cefotaxime

| Recipient | Concentration of Cefotaxime (µg/ml) | Average conjugation frequency | Standard deviation | P value |
|--|-------------------------------------|-------------------------------|-------------------------|---------|
| <i>E. coli</i> J53-2 (rifampicin ^R) | No CTX | 4.88 x 10 ⁻⁵ | 4.82 x 10 ⁻⁵ | |
| | 0.003 | 3.40 x 10 ⁻⁵ | 1.58 x 10 ⁻⁵ | 0.904 |
| | 0.006 | 2.43 x 10 ⁻⁵ | 6.02 x 10 ⁻⁶ | 0.610 |
| <i>E. coli</i> DH5α (rifampicin ^R) | No CTX | 4.87 x 10 ⁻⁵ | 4.26 x 10 ⁻⁵ | |
| | 0.003 | 2.04 x 10 ⁻⁵ | 2.18 x 10 ⁻⁵ | 0.364 |
| | 0.006 | 1.20 x 10 ⁻⁵ | 1.06 x 10 ⁻⁵ | 0.222 |
| <i>E. coli</i> 3950 (tetracycline ^R) | No CTX | 4.00 x 10 ⁻⁶ | 9.84 x 10 ⁻⁷ | |
| | 0.003 | 3.05 x 10 ⁻⁶ | 1.08 x 10 ⁻⁶ | 0.081 |
| | 0.006 | 2.73 x 10 ⁻⁵ | 3.36 x 10 ⁻⁵ | 0.314 |
| <i>S. Typhimurium</i> SL1344 rif ^R (rifampicin ^R) | No CTX | 1.10 x 10 ⁻⁵ | 7.32 x 10 ⁻⁶ | |
| | 0.003 | 1.72 x 10 ⁻⁵ | 8.40 x 10 ⁻⁶ | 0.389 |
| | 0.006 | 1.97 x 10 ⁻⁵ | 6.32 x 10 ⁻⁶ | 0.195 |

The conjugation frequency of pCT in the presence of sub-inhibitory concentration of cefotaxime (CTX) was compared to conjugation frequency in the absence of antibiotic. Frequencies were measured from donor bacterial strain *E. coli* DH5α to recipient strains *E. coli* J53-2, *E. coli* DH5α, *E. coli* 3950 and *S. Typhimurium* SL1344 rif^R on a filter placed on an agar containing either no cefotaxime; 0.003µg/ml cefotaxime or 0.006µg/ml cefotaxime

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Conjugation frequencies were calculated from the mean of at least three separate experiments (Figure 4.9, page 155). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are in bold text.

Appendix 11. Conjugation frequencies in liquid media of pCT in the presence of sub-inhibitory concentrations of cefotaxime

| Recipient | Concentration of Cefotaxime (µg/ml) | Average conjugation frequency | Standard deviation | P value |
|---|-------------------------------------|-------------------------------|-------------------------|---------|
| <i>E. coli</i> J53-2 (rifampicin ^R) | No CTX | 4.88 x 10 ⁻⁵ | 4.82 x 10 ⁻⁵ | |
| | 0.003 | 3.40 x 10 ⁻⁵ | 1.58 x 10 ⁻⁵ | 0.692 |
| | 0.006 | 2.43 x 10 ⁻⁵ | 6.02 x 10 ⁻⁶ | 0.509 |
| <i>E. coli</i> DH5α (rifampicin ^R) | No CTX | 1.47 x 10 ⁻⁴ | 2.01 x 10 ⁻⁴ | |
| | 0.003 | 2.91 x 10 ⁻⁵ | 9.05 x 10 ⁻⁶ | 0.495 |
| | 0.006 | 3.05 x 10 ⁻⁵ | 2.69 x 10 ⁻⁵ | 0.359 |
| <i>E. coli</i> 3950 (tetracycline ^R) | No CTX | 3.07 x 10 ⁻⁵ | 9.99 x 10 ⁻⁷ | |
| | 0.003 | 1.16 x 10 ⁻⁴ | 2.99 x 10 ⁻⁵ | 0.525 |
| | 0.006 | 1.66 x 10 ⁻⁴ | 1.15 x 10 ⁻⁵ | 0.143 |
| <i>S. Typhimurium</i> SL1344 (rifampicin ^R) | No CTX | 6.55 x 10 ⁻⁶ | 4.47 x 10 ⁻⁶ | |
| | 0.003 | 1.01 x 10 ⁻⁵ | 2.26 x 10 ⁻⁶ | 0.225 |
| | 0.006 | 8.90 x 10 ⁻⁶ | 2.95 x 10 ⁻⁶ | 0.405 |

The conjugation frequency of pCT in the presence of sub-inhibitory concentration of cefotaxime (CTX) was compared to conjugation frequency in the absence of antibiotic. Frequencies were measured from donor bacterial strain *E. coli* DH5α to recipient strains *E. coli* J53-2, *E. coli* DH5α, *E.coli* 3950 and *S. Typhimurium* SL1344 rif^R in LB broth containing either no cefotaxime; 0.003µg/ml cefotaxime or 0.006µg/ml cefotaxime.

Conjugation frequencies were calculated from the mean of at least three separate experiments (Figure 4.10, page 156). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant are in bold text.

Appendix 12. DNA sequence showing the successful insertion of the *aph-gfp* construct into *bla*_{CTX-M-14} on pCT (construction of pCT2b)

```

GGCGTGCATTCCGCTGCTGCTGGGCAGCGCGCCGCTTTATGCGCAGACGAGTGCGGTGCAGCAAAAGC
TGGCGGCAGGAGTCCAAGCGAGTCTCGAACCCAGAGTCCCCGCTCAGAAGAAGTTCGTCAAGAAGGC
GATAGAAGGCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCAT
TCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCAACGCTATGTCCTGATAGCGGTCCGCCACACC
CAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGCAT
CGCCATGGGTACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTTCGGCT
GGCGCGAGCCCCTGATGCTCTTCGTCCAGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACG
TGCTCGCTCGATGCGATGTTTCGCTTGGTGGTTCGAATGGGCAGGTAGCCGGATCAAGCGTATGCAGCC
GCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGC
CCCGGCACTTCGCCCAATAGCAGCCAGTCCCTTCCCCTTCAGTGACAACGTCGAGCACAGCTGCGCA
AGGAACGCCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCTGCAGTTTCATTTCAGGGCACCGG
ACAGGTGGTCTTGACAAAAGAACCAGGGCGCCCCTGCGCTGACAGCCGAAACACGGCGGCATCAGAGC
AGCCGATTGTCTGTTGTGCCAGTCATAGCCGAATAGSCTCTCCACCCAAGCGGCGGAGAACCTGCGT
GCATCCATCTTGTTCAATMATGCAACGATCCTCATCCTGTCTCTTGATCAGATCTTGATCCCCTGCS
CATCAGATCCTTGGCGGCAARAAGCCATCCAGTTTACTTTGCAGGCTCCCAACTACARAGGGCGCCCA
GCTGGCATTTCGACGTCTAGAACCATWTWVCWGRAMTTACTWAAATAGCGTWWCMGAGCTTCGCTTMMC
YCRAGTCTGSGTMMCTCGCGCGCCMAGATCGCCACGGTGCATCTGATCGMGAAGMGCTACTMMGGKG
ACCTGCAGTGACSGGGATACTGTCTGACGAATTGCAATGTYCWGTGCSCACTGCGGGTGWGGACATATT
ATTGC.....
.....GTCCMCYGGSTGACACRGATATCACGST
GTMTYCGMKMTCGAGAMGTTYMTSCAGGTACTGCATGAGCGGGATTACTGTRCGGATGCCAAAKTCWG
TGGSCSAKTGSTGTAGATAWATWGSAAKGWTSSTGATGGGTAGGCTCAATACGKAAATCGKGTAGA
CTGCGGATTAGTGCAATTTACATTTWMMTACGAACMTCSGAAGSRGTTGATAGATTAGAGTAKRGCMT
ATCGAGAACGCGTAGAAATATCGSGACAGCTGGSGTAGCAGGAGAGTACCACAATSCTTTCGTGAAG
ACCTGGCGSGGATCTCTAGATTAGAGGAGATATACATWTGAGTAAAGGAGAGACTTTCAGTGGAGTGT
CCCATCTGTGAATAGATGTGATGTTAATGGCACAAATTTCTGTGAGTGGAGAGGKGAAGGTGATGCAA
CATACGGAAGACTTACCCTTAAATTTATTGCACTACTGGAAAACACTACCTGTCCCATGGCCAACACTTGT
CACTACTTTTCGCGTATGGTCTTCAATGCTTGCAGATACCCAGATCATATGAAACAGCATGACTTTTC
AAGAGTGCCATGCCGAAGGTTATGTACAGGAAAGAACTATATTTTTTCAAAGATGACGGGAACACAA
GACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAGGTATTGATT
TTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACATACTACACAAATGTATACATC
ATGGCAGACAAACAAAAGAATGGAATCAAAGTTAACTTCAAATTAGACACAACATTGAAGATGGAAG
CGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCTTTTACCAGACA
ACCATTACCTGTCCACACAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTT
CTTGAGTTTGTAAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAGATGTCCAGACC
TGCAGGCATGCAAGCTCTAGAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTTCGT
TTTATCTGTTGTTTGTGCGGTGAACGCTCTCCTCGACTACGGCACCAATGATATTGCGGTGATCTG
GCCCCAGGGTCGTGCGCCGCTGGTTCTGGTGACCTATTTTACCCAGCCGCAACAGAACGCAGAGAGCC

```

Red text, DNA sequence homologous to the *bla*_{CTX-M-14} gene,

Orange text, sequence homologous to pUA66*pacpP* PCR construct to the *aph* gene,

Green text, sequence homologous to pUA66*pacpP* PCR construct to the *gfp* gene,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 13. DNA sequence showing the successful insertion of the *aph* gene into *ISEcp1* on pCT (construction of pCT6)

CMGRAKYWATGGGGGATTTTGGATTTTATTGAAATGACCTCGTATTTGGATAATGACTCAAC
 AAATAAAATCAAGATGAATCATATAAAGACCATGCTCTGGTGTAGGCTGGAGCTGCTTCGAA
 GTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCAAGATCCCCCACGCTGC
 CGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCA
 GAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAA
 GCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTT
 TTATGGACAGCAAGCGAACCAGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCC
 CTGCAAAGTAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGAT
 CTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGT
 TCTCCGGCCGCTTGGGTGGAGAGGCTATTTCGGCTATGACTGGGCACAACAGACAATCGGCTG
 CTCTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCG
 ACCTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACG
 ACGGGCGTTTCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCT
 ATTTGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATC
 CATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTTCGACC
 ACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAG
 GATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACTGTTTCGCCAGGCTCAAGGC
 GCGCATGCCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCA
 TGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGC
 TATCAGGACATAGCGTTGGCTACCCGTGATATTACTGAAGAGCTTGGCGGCGAATGGGCTGA
 CCGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGAGCGCATCGCCTTCTATCGCC
 TTCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCA
 ACCTGCCATCACGAGATTTTCGATTCCACCGCCGCTTCTATGAAAGGTTGGGCTTCGGAATC
 GTTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGC
 CCACCCCAGCTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACKTCGAA
 TAGGAAC TAAGGAGGATATTCATATGGACCATGGCTAWTCCC AATCAAAACCGCAAGAATWK
 KWWTWYCKSSMYC

Red text, DNA sequence homologous to the *ISEcp1*,

Black text, DNA sequence homologous to the *aph* gene region in L109,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 14. Conjugation frequencies of pCT and *bla*_{CTX-M-14} mutants pCT2, pCT2b and pCT6 on solid media to various recipients

Donor: *E. coli* DH5 α , Recipient: *S. Typhimurium* SL1344 rif^R

| | Average frequency | SD | Student's T-test value (<i>P</i>) |
|-------|-------------------------|-------------------------|-------------------------------------|
| pCT | 5.95 x 10 ⁻⁶ | 5.39 x 10 ⁻⁶ | |
| pCT2 | 2.27 x 10 ⁻⁵ | 9.34 x 10 ⁻⁶ | 0.0525 |
| pCT2b | 3.38 x 10 ⁻⁶ | 5.74 x 10 ⁻⁶ | 0.6018 |
| pCT6 | 2.28 x 10 ⁻⁵ | 1.18 x 10 ⁻⁵ | 0.0876 |

Donor: *E. coli* DH5 α , Recipient: *E. coli* J53-2

| | Average frequency | SD | Student's T-test value (<i>P</i>) |
|-------|-------------------------|-------------------------|-------------------------------------|
| pCT | 1.70 x 10 ⁻² | 1.41 x 10 ⁻² | |
| pCT2 | 3.40 x 10 ⁻² | 2.28 x 10 ⁻² | 0.226 |
| pCT2b | 3.41 x 10 ⁻⁴ | 1.88 x 10 ⁻⁴ | 0.144 |
| pCT6 | 2.74 x 10 ⁻² | 2.74 x 10 ⁻² | 0.066 |

Donor: *E. coli* DH5 α , Recipient: *E. coli* 3950

| | Average frequency | SD | Student's T-test value (<i>P</i>) |
|-------|-------------------------|-------------------------|-------------------------------------|
| pCT | 6.12 x 10 ⁻⁶ | 1.27 x 10 ⁻⁶ | |
| pCT2 | 8.71 x 10 ⁻⁶ | 6.86 x 10 ⁻⁶ | 0.555 |
| pCT2b | 1.55 x 10 ⁻⁵ | 2.53 x 10 ⁻⁵ | 0.556 |
| pCT6 | 7.51 x 10 ⁻⁶ | 1.30 x 10 ⁻⁵ | 0.162 |

The conjugation frequency of pCT2 (*bla*_{CTX-M-14::aph}), pCT2b (*bla*_{CTX-M-14::aph-gfpmut2}) and pCT6 (*ISEcp1::aph*) were compared to the conjugation frequency of wild-type pCT from donor bacterial strain *E. coli* DH5 α to recipient strains *S. Typhimurium* SL1344 rif^R, *E. coli* J53-2 and *E. coli* 3950 on a filter.

Conjugation frequencies were calculated from the mean of at least three separate experiments (Figure 5.5, page 182). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are in bold text.

Appendix 15. Conjugation frequencies of pCT and *bla*_{CTX-M-14} mutants pCT2, pCT2b and pCT6 in liquid media to various recipients

Donor: *E. coli* DH5 α , Recipient: *S. Typhimurium* SL1344 rif^R

| | Average frequency | SD | Student's T-test value (<i>P</i>) |
|-------|-------------------------|-------------------------|-------------------------------------|
| pCT | 5.25 x 10 ⁻⁶ | 5.92 x 10 ⁻⁶ | |
| pCT2 | 1.5 x 10 ⁻⁵ | 1.89 x 10 ⁻⁵ | 0.179 |
| pCT2b | 3.09 x 10 ⁻⁶ | 3.16 x 10 ⁻⁶ | 0.699 |
| pCT6 | 3.74 x 10 ⁻⁵ | 2.98 x 10 ⁻⁵ | 0.0725 |

Donor: *E. coli* DH5 α , Recipient: *E. coli* J53-2

| | Average frequency | SD | Student's T-test value (<i>P</i>) |
|-------|--------------------------|-------------------------|-------------------------------------|
| pCT | 7.28 x 10 ⁻⁵ | 6.14 x 10 ⁻⁵ | |
| pCT2 | 2.59 x 10 ⁻⁴ | 3.29 x 10 ⁻⁴ | 0.309 |
| pCT2b | 5.45 x 10 ⁻⁶ | 5.57 x 10 ⁻⁶ | 0.069 |
| pCT6 | 1.991 x 10 ⁻³ | 1.95 x 10 ⁻³ | 0.098 |

Donor: *E. coli* DH5 α , Recipient: *E. coli* 3950

| | Average frequency | SD | Student's T-test value (<i>P</i>) |
|-------|--------------------------|-------------------------|-------------------------------------|
| pCT | 1.394 x 10 ⁻⁵ | 1.52 x 10 ⁻⁴ | |
| pCT2 | 3.14 x 10 ⁻⁴ | 5.08 x 10 ⁻³ | 0.177 |
| pCT2b | 2.90 x 10 ⁻⁴ | 3.13 x 10 ⁻⁴ | 0.411 |
| pCT6 | 3.327 x 10 ⁻³ | 3.62 x 10 ⁻³ | 0.0835 |

The conjugation frequency of pCT2 (*bla*_{CTX-M-14::aph}), pCT2b (*bla*_{CTX-M-14::aph-gfpmut2}) and pCT6 (*ISEcp1::aph*) were compared to the conjugation frequency of wild-type pCT from donor bacterial strain *E. coli* DH5 α to recipient strains *S. Typhimurium* SL1344 rif^R, *E. coli* J53-2 and *E. coli* 3950 in liquid media.

Conjugation frequencies were calculated from the mean of at least three separate experiments (Figure 5.6, page 183). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted in bold text.

Appendix 16. Generation times of *E. coli* 3950 containing plasmids pCT2, pCT2b and pCT6 compared to *E. coli* 3950 pCT

| Lab code | Strain | Generation time (mins) | SD | Student T-test (<i>P</i>) |
|----------|--|------------------------|-------|-----------------------------|
| I855 | <i>E. coli</i> 3950 pCT | 68.73 | 5.527 | |
| I835 | <i>E. coli</i> 3950 pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 69.21 | 5.129 | 0.377 |
| I836 | <i>E. coli</i> 3950 pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfp</i>) | 68.26 | 5.958 | 0.600 |
| I838 | <i>E. coli</i> 3950 pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 68.20 | 5.933 | 0.234 |

The growth kinetics of bacterial host strain *E. coli* 3950 containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Figure 5.7, page 185). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted in bold text.

Appendix 17. Generation times of *E. coli* J53-2 containing plasmids pCT2, pCT2b and pCT6 compared to *E. coli* J53-2 pCT

| Lab code | Strain | Generation time (mins) | SD | Student T-test (<i>P</i>) |
|----------|---|------------------------|------|-----------------------------|
| l834 | <i>E. coli</i> J53-2 pCT | 67.14 | 3.75 | |
| l856 | <i>E. coli</i> J53-2 pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 63.28 | 5.84 | 0.952 |
| l857 | <i>E. coli</i> J53-2 pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfp</i>) | 67.30 | 2.06 | 0.334 |
| l861 | <i>E. coli</i> J53-2 pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 71.19 | 5.66 | 0.057 |

The growth kinetics of bacterial host strain *E. coli* J53-2 containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Figure 5.8, page 186). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted in bold text.

Appendix 18. Generation times of *E. coli* DH5α containing plasmids pCT2, pCT2b and pCT6 compared to *E. coli* DH5α pCT

| Lab code | Strain | Generation time (mins) | SD | Student T-test (<i>P</i>) |
|----------|--|------------------------|-------------|-----------------------------|
| I755 | <i>E. coli</i> DH5α pCT | 92.61 | 6.70 | |
| I778 | <i>E. coli</i> DH5α pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 95.15 | 9.06 | 0.94 |
| I826 | <i>E. coli</i> DH5α pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfp</i>) | 79.80 | 6.56 | 0.0198 |
| I830 | <i>E. coli</i> DH5α pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 99.64 | 6.03 | 0.121 |

The growth kinetics of bacterial host strain *E. coli* DH5α containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfp**mut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Figure 5.9, page 187). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted in bold text.

Appendix 19. Generation times of *S. Typhimurium* rif^R containing plasmids pCT2, pCT2b and pCT6 compared to *S. Typhimurium* rif^R SL1344 pCT

| Lab code | Strain | Generation time (mins) | SD | Student T-test (<i>P</i>) |
|----------|---|------------------------|--------------|-----------------------------|
| L1079 | <i>S. Typhimurium</i> SL1344 rif ^R pCT | 95.15 | 11.7 | |
| L1244 | <i>S. Typhimurium</i> SL1344 rif ^R pCT2 (<i>bla</i> _{CTX-M-14} :: <i>aph</i>) | 100.73 | 9.77 | 0.278 |
| L1235 | <i>S. Typhimurium</i> SL1344 rif ^R pCT2b (<i>bla</i> _{CTX-M-14} :: <i>aph-gfp</i>) | 102.73 | 10.52 | 0.145 |
| L1245 | <i>S. Typhimurium</i> SL1344 rif ^R pCT6 (<i>ISEcp1</i> :: <i>aph</i>) | 110.91 | 16.74 | 0.036 |

The growth kinetics of bacterial host strain *S. Typhimurium* SL1344 rif^R containing either pCT, pCT2 (*bla*_{CTX-M-14}::*aph*), pCT2b (*bla*_{CTX-M-14}::*aph-gfpmut2*) or pCT6 (*ISEcp1*::*aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Figure 5.10, page 188). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted in bold text.

Appendix 20. The ability of *S. Typhimurium* SL1344 rif^R with wild-type pCT and *bla*_{CTX-M-14} modified plasmids to adhere to and invade human intestinal cells

| | SL1344 rif ^R pCT (L1079) | SL1344 rif ^R pCT2 (L1244) | SL1344 rif ^R pCT2b (L1235) | SL1344 rif ^R pCT6 (L1245) | SL1344 rif ^R (L1078) |
|---|---|--|---|--|------------------------------------|
| A: Adhesion (% of SL13444 rif ^R pCT) | 100% | 168% | 23% | 54.67% | 63.3% |
| B: Invasion (% of SL13444 rif ^R pCT) | 100% | 93.25% | 22% | 18.47% | 43% |

The number of *S. Typhimurium* SL1344 rif^R containing pCT2 pCT2b or pCT6 able to adhere to (A) and invade (B) INT-407 human intestinal cell is represented as a percentage of the number of *S. Typhimurium* SL1344 rif^R pCT able to do so under test conditions (Figure 5.11, p190). Data are displayed as a mean of three separate experiments all performed with four technical repeats.

Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are represented with bold text.

Appendix 21. The ability of bacterial hosts containing pCT2, pCT2b and pCT6 to cause infection the *C. elegans*

| | Lab no | Strain | TD ₅₀ (Days) | Chi squared | P value |
|----------|--------|---|-------------------------|--------------|---------------|
| A | L1079 | <i>S. Typhimurium</i> SL1344 rif ^R pCT | 3.15 | | |
| | L1244 | <i>S. Typhimurium</i> SL1344 rif ^R pCT2 | 3.5 | 1.6 | 0.2059 |
| | L1235 | <i>S. Typhimurium</i> SL1344 rif ^R pCT2b | 3.0 | 0.017 | 0.8963 |
| | L1245 | <i>S. Typhimurium</i> SL1344 rif ^R pCT6 | 3.8 | 15.09 | 0.0001 |
| B | I855 | <i>E. coli</i> 3950 pCT | 5.75 | | |
| | I835 | <i>E. coli</i> 3950 pCT2 | 5.75 | 0.294 | 0.5877 |
| | I838 | <i>E. coli</i> 3950 pCT6 | 5.6 | 0.3518 | 0.5531 |

Survival curves were constructed using a Kaplan Mier calculation showing the probability of *C. elegans* survival each day when fed continuously on the tested bacterial strains (Figure 5.12, page 191).

TD₅₀ = time taken for 50% of the nematodes to die,

A significance difference in killing rate was defined as a P value <0.05 denoted in bold text.

Appendix 22. Competition between *E. coli* hosts carrying pCT and pCT2

| <i>E. coli</i> pairing | pCT2 CI at 12h (before passage) | pCT2 CI at 72h/96h (after 3/4 passages) | T-test P value |
|------------------------|------------------------------------|--|-------------------|
| DH5α pCT + DH5α pCT2 | 0.989 | 1.000 | 0.477 |
| 3950 pCT + 3950 pCT2 | 1.005 | 0.999 | 0.217 |
| J53-2 pCT + J53-2 pCT2 | 1.003 | 1.071 | 0.038 |

CI, competition index of the pCT containing strain in each pairing. Values in bold represent a statistically significant change in the proportion of bacteria containing pCT ($P < 0.005$).

Table of data corresponding with Figure 5.13 (page 193).

Appendix 23. DNA sequence showing the successful insertion of the *aph* gene into pCT_006 putative Sigma factor gene on pCT (construction of pCT3)

```

CTCAAACACATCAAGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAAAYAGRAACTTC
GGAATAGGAACTTCAAGATCCCCCACGCTGCCGCAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAGC
GGAACACGTAGAAAGCCAGTCCGCAGAAACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATC
TGGACAAGGGAAAACGCAAGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCT
AGACTGGGCGGTTTTATGGACAGCAAGCGAACCGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTG
GGAAGCCCTGCAAAGTAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGA
TCTGATCAAGAGACAGGATGAGGATCGTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCC
GGCCGCTTGGGTGGAGAGGCTATTTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGATGCCG
CCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGTCCGGTGCCCTG
AATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCACGACGGGCGTTTCTTGCGCAGCTGTG
CTCGACGTTGTCACTGAAGCGGGAAGGACTGGCTGCTATTGTGCGAAGTGCCGGGGCAGGATCTCCT
GTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCATGGCTGATGCAATGCGGCGGCTGCATACGC
TTGATCCGGCTACCTGCCCATTTCGACCACCAAGCGAAACATCGCATCGAGCGAGCACGTACTCGGATG
GAAGCCGGTCTTGTCGATCAGGATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAACGTGT
CGCCAGGCTCAAGGCGCGCATGCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGC
CGAATATCATGGTGGAAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGAC
CGCTATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCG
CTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGCAGCGCATCGCCTTCTATCGCCTTCTTGACG
AGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCAACCTGCCATCACGAG
ATTTGCAATTCCACCGCCGCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTTCCGGGACGCCGGCTGG
ATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCCACCCAGCTTCAAAAGCGCTCTGAA
GTTCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTAAGGAGGATATTCATATGGACCATG
GCTAATTCCCACCGAATGACCTTGRGTCTCACGAGAATAAAAAGTAATGCAGGTCGCAAATGGGCACA
TACTCGAGGATGGGGATGGTTGTCATATGAA

```

Red text, DNA sequence homologous to the pCT_066 putative Sigma factor gene,

Black text, DNA sequence homologous to the *aph* gene region in L109,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 24. DNA sequence showing the successful insertion of the *aph* gene into shufflon recombinase (*rci*) gene on pCT (construction of pCT4)

```

AYGTCACCCTATGGCCGAGCGCTATATGGATGAAATCACCACCGTTGATATCG
CCACTTACCGGGATCAGCGTTTGGCTCAAATTAACCCACGAACAGGGCGTCAA
ATCACCGGAAACACCGTCCGTCTTGAACTTGCTCTTTTATMATCATTATTCAA
TATCGCCAGTGTGGAATGGGGGACATGCCGTATGAATCCTGTTGAACTGGTCC
GAAAGTGTAGGCTGGAGCTGCTTCGAAGTTCCTATACTTTCTAGAGAATAGGA
ACTTCGGAATAGGAACTTCAAGATCCCCCACGCTGCCGCAAGCACTCAGGGCG
CAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACGGT
GCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCA
AGCGCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGA
CTGGGCGGTTTTTATGGACAGCAAGCGAACCAGGAATTGCCAGCTGGGGCGCCCT
CTGGTAAGGTTGGGAAGCCCTGCAAAGTAAACTGGATGGCTTTCTTGCCGCCA
AGGATCTGATGGCGCAGGGGATCAAGATCTGATCAAGAGACAGGATGAGGATC
GTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCGGCCGCTTGGG
TGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGAT
GCCGCCGTGTTCCGGCTGTCAGCGCAGGGCGCCCGGTTCTTTTTGTCAGACCG
ACCTGTCCGGTGCCCTGAATGACTGCAGGACGAGGCAGCGCGGCTATCGKGCT
GGCMCGACGGGCGTTCCTTGCGCAGCTGTGCTCGAC

```

Red text, DNA sequence homologous to the shufflon recombinase (*rci*) gene,

Black text, DNA sequence homologous to the *aph* gene region in L109,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 25. DNA sequence showing the successful insertion of the *aph* gene into pili structural protein *pilS* gene on pCT (construction of pCT5)

```

TTAACTGYMTGGTTTCCAGTTTGATAGTAGTTTTATCTGCGTTACATACTGTAGACGGGTCA
ACTTTATTTCGTCACATTGCCGTTGATTTTTGTAAACATGGATGAACTGCGCAGGCTGGTGAC
CAGATCAATACAGTTTTTCTTCGGCACATTGCTGTGTAGGCTGGAGCTGCTTCGAAGTTCCT
ATACTTTTAGAGAATAGGAACTTCGGAATAGGAACTTCAAGATCCCCTCACGCTGCCGCAAG
CACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGAAACG
GTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGCGCAA
AGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTTATGG
ACAGCAAGCGAACCAGGAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCTGCAA
AGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCTGATC
AAGAGACAGGATGAGGATCGTTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTCTCCG
GCCGCTTGGGTGGAGAGGCTATTCGGCTATGACTGGGCACAACAGACAATCGGCTGCTCTGA
TGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGACCTGT
CCGGTGCCCTGAATGAACTGCAGGACGAGGCAGCGCGGCTATCGTGGCTGGCCACGACGGGC
GTTCCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTATTGGG
CGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCCATCA
TGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCACCAA
GCGAAACATCGCATCGAGCGAGCACGTACTCGGATGGAAGCCGGTCTTGTCGATCAGGATGA
TCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAGTGTTCGCCAGGCTCAAGGCGCGCA
TGCCCCGACGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCATGGTG
GAAATGGCCGCTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCTATCA
GGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGACCGCT
TCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGACGCGCATCGCCTTCTATCGCCTTCTT
GACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCAACCTG
CCATCACGAGATTTTCGATTCCACCGCCGCTTCTATGAAAGGTTGGGCTTCGGAATCGTTTT
CCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCCACC
CCAGCTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGA
ACTAAGGAGGATTTTCATATGGACCATGGCTAATTCCCTTTCTGGGCTTTCATTTTGGTGGCG
ATTGTCTGCAGGTTTTTGAGTTCCTTCTGGTTGTCTGSAGAGGACAGGGTATCGGACATAAG
TCCCATCACGGCACCAATAACAATGGCAMCGACGACCAGAACACCGAGGACTTCCAGCAGGG
TCATGCCTTTATCATCGTM

```

Red text, DNA sequence homologous to the *pilS* gene,

Black text, DNA sequence homologous to the *aph* gene region in L109,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 26. DNA sequence showing the successful insertion of the *aph* gene into pili structural protein *traXY* gene on pCT (construction of pCT7)

```

GAAAGGGGGGAGGACCAATSTAATGACGGATTCCGAAC TGGGCCGGCGGCTGCGACGTCACA
CGATGTTTTTGGTCGCCWGGATTTTTTTTTTGATSACGAGACCGGTATTCCTGCTGTGTGGTATG
GCCCTTCGGCCTCCGCACTGCCGGTCATTCCCCTGATAC TTATCCTGACCTGCCTGTTACT
GATGAKTACCATTGCKCGTG TARGCTGGAKCTGCTTC KAAKTTCTTATACTTTCTARAKAAT
AGGAWCTTCSGAATAKGAAC TTCAMGATCCCCCACKCYGCCCKCAWGC ACTCAKGGCGCAAGG
GCTGCTARATGAAGCGGAWTACKTATAAAGCCWKTTCGCRYAAACGGTGCTGACCCCGGATG
ATTGTCWGTATTGGGCTATCTGGATAGGGAAAACGCACYCGCAAAGACAAAGCAGGTAGCT
TGCACTGGGCTTACATGGCSATATCTASACTGGGCGGTTTTATGGACARCMMGCRAACCGGA
ATTGCCAGCSGGGGCGCCCTCTGGTAAYGTTGGGA AKCCCTGCMAWGTAAWTGGATGGCTTT
CTTGCCSCCAAGGATCTSATGGCGCATAGGATCMMKATCTGATCAWGA KACARGATGAGGAT
CGTTCGCATGATTGAAAATG GATTGACACGYAGTTCTCCKGCCGCTTGGSTGGAGAGGCYA
TTCSGCTATGACTGGWCWCRACARACAATCRGCTGCTCTTGATGCCGCCTGTTCCGGCTGTC
WGCGCATGGGCGCTCGGTTCTTTTTTGCTMGACCGACCTGYCCSGYGCCCTGAATGAAC TGCA
GACTACGCAGCGCGKCTATC-TGGCTGGCCACGACGG-CGTTCTTGCCYATCTGTGCTC-
ACKTTGTCCTGAKCKGAWGGACTGKCTGCTATTGGCKATTGCCGKGCM SGATCTCCTGTC-
TCTCAC..TTGCTC..TGY.....GAAAARKATCCATCATGCTGATGCCATGTGC.....
.....GCTGMACGCTTGWWCCGKCTACCTGCCATTY.....
.....WMCWCCAGGGTATCGCCGCTCCMGATTTCGCAGCGCATMGCA
TTSTASGTTTCTGACGAGTTYTTMTGAGCGGGACTAGGGGTKMGAAAYGACMGASCAAGCGA
CGCCCAACCTACCATCACGAGATTTKGATTCCACCGCCGCCWTCTAWGAAAGKYTG GGSTTM
GGAGTMGTTTTCCGGGATGCMGWCTGGATGATCCTCCAGCGCGGGGATWTCATRCTGGAGTT
MTTCGCCACCCACGCTTCAAAGCGMTMTGAAGTTCMTATACTTTYTAGAGAATAGGAACT
TCGGAATAGGAAMTAAGGAGGATATTCAT ATGGACCATGGCTAAT TCCCCCACC ACTSTSCT
TCCTGCWGTTTTKGCTGTTTTGTGCCGGTTTTTCGTTATCCATTTACCKGCCGTTWATCCSG
TTWATMTTCWGGATGACCGGCATCGGCAACWGGATAGTCAGMGTAMTGAWWGGAKGTGWACT
GGCCCCCTGWGGGCCGCCACCCATSWGGGAACAAGTCAGGACCGGGGAAGCCGGGCCG MTTA
CGKTTACAATACGTGATRGACAGCATGATCAGGCCGATGGAGAACGTGTTTGGGTTCGTGGW
TGWATCAGTCGCCATCGKGGCCGGCGGCACAATCMTGAAAAGGGGYTATGSKAWGCAMWGAT
GAAKGTCCAGATAAASTTGAGGGGGTAATCAAKCATGGGRGGAATTTTTATTAAAGTTTGA

```

Red text, DNA sequence homologous to the *traXY* genes,

Black text, DNA sequence homologous to the *aph* gene region in L109,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 27. DNA sequence showing the successful insertion of the *aph* gene into putative toxin anti-toxin operon *pndACB* on pCT (construction of pCT8)

```

GCGGCGGGCGCTTGAGGCTGTATGCCGAAAGCGTTTTGTGGACGGCATGTAGCAGAAAGCCC
CAGGCGATTTTTTTTATCAATCAACCCAGGGCCCACTGTGTGTAGGCTGGAGCTGCTTCGAAG
TTCCTATACTTTCTAGAGAATAGGAAC TTCGAATAGGAAC TTCAGATCCCCACGCTGCCG
CAAGCACTCAGGGCGCAAGGGCTGCTAAAGGAAGCGGAACACGTAGAAAGCCAGTCCGCAGA
AACGGTGCTGACCCCGGATGAATGTCAGCTACTGGGCTATCTGGACAAGGGAAAACGCAAGC
GCAAAGAGAAAGCAGGTAGCTTGCAGTGGGCTTACATGGCGATAGCTAGACTGGGCGGTTTT
ATGGACAGCAAGCGAACCGBAATTGCCAGCTGGGGCGCCCTCTGGTAAGGTTGGGAAGCCCT
GCAAAGTAAACTGGATGGCTTTCTTGCCGCCAAGGATCTGATGGCGCAGGGGATCAAGATCT
GATCAAGAGACAGGATGAGGATCGTTTTCGCATGATTGAACAAGATGGATTGCACGCAGGTTC
TCCGGCCGCTTGGGTGGAGAGGCTATTCCGGCTATGACTGGGCACAACAGACAATCGGCTGCT
CTGATGCCGCCGTGTTCCGGCTGTCAGCGCAGGGGCGCCCGGTTCTTTTTGTCAAGACCGAC
CTGTCCGGTGCCCTGAATGAACTGCAGGACGAGGACGCGCGGCTATCGTGGCTGGCCACGAC
GGGCGTTCCTTGCGCAGCTGTGCTCGACGTTGTCACTGAAGCGGGAAGGGACTGGCTGCTAT
TGGGCGAAGTGCCGGGGCAGGATCTCCTGTCATCTCACCTTGCTCCTGCCGAGAAAGTATCC
ATCATGGCTGATGCAATGCGGCGGCTGCATACGCTTGATCCGGCTACCTGCCCATTCGACCA
CCAAGCGAAACATCGCATCGAGCGAGCACGTA CTGGATGGAAGCCGGTCTTGTCGATCAGG
ATGATCTGGACGAAGAGCATCAGGGGCTCGCGCCAGCCGAAC TGTCGCCAGGCTCAAGGCG
CGCATGCCCAGCGGCGAGGATCTCGTCGTGACCCATGGCGATGCCTGCTTGCCGAATATCAT
GGTGGAAAATGGCCGCTTTTTCTGGATTTCATCGACTGTGGCCGGCTGGGTGTGGCGGACCGCT
ATCAGGACATAGCGTTGGCTACCCGTGATATTGCTGAAGAGCTTGGCGGCGAATGGGCTGAC
CGCTTCCTCGTGCTTTACGGTATCGCCGCTCCCGATTTCGCAGCGCATCGCCTTCTATCGCCT
TCTTGACGAGTTCTTCTGAGCGGGACTCTGGGGTTCGAAATGACCGACCAAGCGACGCCCAA
CCTGCCATCACGAGATTTGATTCCACCGCCGCCTTCTATGAAAGGTTGGGCTTCGGAATCG
TTTTCCGGGACGCCGGCTGGATGATCCTCCAGCGCGGGGATCTCATGCTGGAGTTCTTCGCC
CACCCAGCTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAAC TTCGAAT
AGGAACTAAGGAGGATATTCATATGGACCATGGCTAATTCCCCGGACATCTGCCGCCGGRTTG
TTGCGAAGGCTGACCTCAATGCGCCTTGTTGTCTTTTTTACCGGACGACTGCTGGTTGCTG

```

Red text, DNA sequence homologous to the *pndACB* operon,

Black text, DNA sequence homologous to the *aph* gene region in L109,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 28. DNA sequence showing the successful insertion of the *cat* gene into putative toxin anti-toxin operon *pndACB* on pCT7 (construction of pCT9)

```

GGGCGCTTGAGGCTGTATGCCGAAAGCGTTTTGTGGACGGCATGTAGCAGAAAGCCCCAGGC
GATTTTTTTAWMAATCAACCCAGGGCCCACTGTGTGTAGGCTGGAGCTGCTTCGAAGTTCCT
ATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCATTTAAATGGCGCGCCTTACGCC
CCGCCCTGCCACTCATCGCAGTACTGTTGTATTCAATTAAGCATCTGCCGACATGGAAGCCAT
CACAAACGGCATGATGAACCTGAATCGCCAGCGGCATCAGCACCTTGTCGCCTTGCGTATAA
TATTTGCCCATGGTGAAAACGGGGGCGAAGAAGTTGTCCATATTGGCCACGTTTAAATCAAA
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GGAAATAGGCCAGGTTTTTCACCGTAACACGCCACATCTTGCGAATATATGTGTAGAACTGC
CGGAAATCGTCGTGGTATTCACTCCAGARCGATGAAAACGTTTCAGTTTGCTCATGGAAAAC
GGTGTAACAAGGGTGAACACTATCCCATATCACCAGCTCACCGTCTTTCATTGCCATACGTA
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GTGCCGATCAACGTCTCATTTTCGCCAAAAGTTGGCCCAGGGCTTCCCGGTATCAACAGGGA
CACCAGGATTTATTTATTCTGCGAAGTGATCTTCCGTACAGGTAGGCGCGCCGAAGTTCCT
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GCTAATTCCCGGACATCTGCCGCGGRTTGTTGCGAAGGCTGACCTCAATGCGCCTTGTTTG
TCTTTTTTACCGGACGACTGCTGGT-GCTGCG--TCGGC

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Red text, DNA sequence homologous to the *pndACB* operon,

Black text, DNA sequence homologous to the *cat* gene region in L829,

Blue highlighted text, DNA region where homologous recombination has taken place.

Appendix 29. Conjugation frequencies of plasmids from donor *E. coli* DH5α to recipients *E. coli* J53-2, *E. coli* 3950 and *S. Typhimurium* SL1344

| Recipient | Plasmid | Filter mating | | | Broth mating | | |
|---|---------|-------------------------------|--------------------|------|-------------------------------|--------------------|------|
| | | Average conjugation frequency | Standard deviation | P= | Average conjugation frequency | Standard deviation | P= |
| <i>E. coli</i> J53-2 (rifampicin ^R) | pCT | 1.70E-02 | 1.66E-02 | | 9.10E-05 | 6.07E-05 | |
| | pCT2 | 3.40E-02 | 2.29E-02 | 0.36 | 3.34E-04 | 3.58E-04 | 0.31 |
| | pCT3 | 4.81E-02 | 2.61E-02 | 0.16 | 4.51E-03 | 7.36E-03 | 0.36 |
| | pCT4 | 3.27E-02 | 4.27E-02 | 0.58 | 4.59E-04 | 5.17E-04 | 0.29 |
| | pCT5 | 1.28E-01 | 1.49E-01 | 0.27 | 2.96E-03 | 4.95E-03 | 0.37 |
| | pCT7 | 0 | 0 | 0.15 | 0 | 0 | 0.06 |
| | pCT8 | 1.13E-02 | 2.55E-03 | 0.59 | 1.44E-05 | 4.75E-06 | 0.09 |
| | pCT9 | 0 | 0 | 0.15 | 0 | 0 | 0.06 |
| <i>E. coli</i> 3950 (tetracycline ^R) | pCT | 6.12E-06 | 1.27E-06 | | 1.39E-04 | 1.52E-04 | |
| | pCT2 | 8.71E-06 | 6.86E-06 | 0.55 | 3.14E-03 | 4.08E-03 | 0.17 |
| | pCT3 | 5.23E-04 | 8.21E-04 | 0.34 | 3.23E-03 | 4.01E-03 | 0.12 |
| | pCT4 | 1.20E-04 | 2.02E-04 | 0.38 | 5.44E-03 | 1.19E-02 | 0.36 |
| | pCT5 | 5.97E-06 | 9.39E-06 | 0.97 | 6.73E-08 | 9.57E-08 | 0.11 |
| | pCT7 | 0 | 0 | 0.00 | 0 | 0 | 0.11 |
| | pCT8 | 2.10E-04 | 2.40E-05 | 0.21 | 5.24E-03 | 5.50E-04 | 0.06 |
| | pCT9 | 0 | 0 | 0.00 | 0 | 0 | 0.11 |
| <i>S. Typhimurium</i> SL1344 (rifampicin ^R) | pCT | 1.07E-05 | 6.92E-06 | | 9.33E-06 | 7.69E-06 | |
| | pCT2 | 6.85E-04 | 1.16E-03 | 0.37 | 7.14E-04 | 1.13E-03 | 0.34 |
| | pCT3 | 4.87E-05 | 3.93E-05 | 0.17 | 1.20E-04 | 1.21E-04 | 0.19 |
| | pCT4 | 6.162E-07 | 6.00E-07 | 0.06 | 6.87E-09 | 1.19E-08 | 0.10 |
| | pCT5 | 3.93E-06 | 6.55E-06 | 0.29 | 7.69E-08 | 8.02E-08 | 0.10 |
| | pCT7 | 0 | 0 | 0.05 | 0 | 0 | 0.10 |
| | pCT8 | 2.54E-06 | 9.99E-07 | 0.12 | 2.85E-06 | 1.50E-06 | 0.23 |
| | pCT9 | 0 | 0 | 0.05 | 0 | 0 | 0.10 |

Appendix 30. Generation times of *E. coli* DH5α containing pCT, pCT3-9

| Lab code | Strain | Generation time (mins) | SD | Student's T-test (<i>P</i>) |
|----------|---|------------------------|------|-------------------------------|
| I755 | <i>E. coli</i> DH5α pCT | 67.27 | 6.62 | |
| I846 | <i>E. coli</i> DH5α pCT3 (Sigma factor:: <i>aph</i>) | 59.74 | 5.33 | 0.097 |
| I877 | <i>E. coli</i> DH5α pCT4 (<i>rci</i> :: <i>aph</i>) | 59.92 | 7.46 | 0.078 |
| I878 | <i>E. coli</i> DH5α pCT5 (<i>pilS</i> :: <i>aph</i>) | 67.34 | 10.0 | 0.360 |
| I846 | <i>E. coli</i> DH5α pCT7 (<i>traXY</i> :: <i>aph</i>) | 72.71 | 10.5 | 0.066 |
| I877 | <i>E. coli</i> DH5α pCT8 (<i>pndACB</i> :: <i>aph</i>) | 75.97 | 12.1 | 0.067 |
| I878 | <i>E. coli</i> DH5α pCT9 (<i>traXY</i> :: <i>aph</i> ; <i>pndACB</i> :: <i>cat</i>) | 61.89 | 12.3 | 0.473 |

The growth kinetics of bacterial host strain *E. coli* DH5α containing either pCT, pCT3 (Sigma factor::*aph*), pCT4 (*rci*::*aph*), pCT5 (*pilS*::*aph*), pCT7 (*traXY*::*aph*), pCT8 (*pndACB*::*aph*) or pCT9 (*traXY*::*aph*; *pndACB*::*cat*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 2-3 (Figure 6.3, page 219). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted in bold text.

Appendix 31. Generation times of host strains containing pCT, pCT3-5

| Lab code | Strain | Generation time (mins) | SD | Student T-test (<i>P</i>) |
|----------|--|------------------------|-------------|-----------------------------|
| I847 | <i>E. coli</i> J53-2 pCT | 67.14 | 3.75 | |
| I858 | <i>E. coli</i> J53-2 pCT3 | 69.23 | 3.87 | 0.173 |
| I859 | <i>E. coli</i> J53-2 pCT4 | 64.78 | 3.76 | 0.64 |
| I860 | <i>E. coli</i> J53-2 pCT | 65.00 | 2.01 | 0.51 |
| I834 | <i>E. coli</i> 3950 pCT | 67.93 | 5.527 | |
| I837 | <i>E. coli</i> 3950 pCT3 | 70.34 | 3.32 | 0.21 |
| I831 | <i>E. coli</i> 3950 pCT4 | 66.10 | 4.60 | 0.63 |
| I841 | <i>E. coli</i> 3950 pCT5 | 67.67 | 2.58 | 0.42 |
| L1079 | <i>S. Typhimurium</i> SL1344 rif ^R pCT | 94.15 | 11.7 | |
| L1236 | <i>S. Typhimurium</i> SL1344 rif ^R pCT3 | 114.6 | 22.4 | 0.02 |
| L1259 | <i>S. Typhimurium</i> SL1344 rif ^R pCT4 | 116.04 | 43.7 | 0.09 |
| L1260 | <i>S. Typhimurium</i> SL1344 rif ^R pCT5 | 108.28 | 15.25 | 0.06 |

The growth kinetics of bacterial host strains containing either pCT, pCT3 (*Sigma factor::aph*), pCT4 (*rci::aph*) or pCT5 (*pilS::aph*) were measured using the FLUOstar OPTIMA using at least three biological and four technical repeats. Generation times were calculated between hours 1-2 (Figure 6.4, page 220). Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are denoted in bold text.

Appendix 32. The ability of *S. Typhimurium* SL1344 rif^R containing wild-type pCT, pCT3, pCT4, pCT5 and pCT8 to adhere to and invade human intestinal cells

| | SL1344 rif ^R pCT (L1079) | SL1344 rif ^R pCT3 (L1236) | SL1344 rif ^R pCT4 (L1259) | SL1344 rif ^R pCT5 (L1260) | SL1344 rif ^R pCT8 (L1397) |
|--------------------|---|--|--|--|--|
| A: Adhesion | 100% | 50.5% | 32.2% | 55.5% | 38.5% |
| B: Invasion | 100% | 43.58% | 8.17% | 48.19% | 4.05% |

The number of *S. Typhimurium* SL1344 rif^R containing pCT3 pCT4, pCT5 or pCT8 able to adhere to (A) and invade (B) INT-407 human intestinal cell is represented as a percentage of the number of *S. Typhimurium* SL1344 rif^R pCT able to do so under test conditions (Figure 6.5, page 222). Data are displayed as a mean of three separate experiments all performed with four technical repeats.

Values returning a *P* value of <0.05 from a Student's T-test were considered statistically significant and are represented with bold text.

Appendix 33. The ability of bacterial hosts containing pCT3, pCT4, pCT5 and pCT8 to cause infection in *C. elegans*

| Lab number | Strain | TD ₅₀ (Days) | Chi squared | P value |
|------------|--|-------------------------|-------------|------------------|
| L1079 | <i>S. Typhimurium</i> SL1344 rif ^R pCT | 3.15 | | |
| L1078 | <i>S. Typhimurium</i> SL1344 rif ^R | 3.5 | 3.39 | 0.0658 |
| L1236 | <i>S. Typhimurium</i> SL1344 rif ^R pCT3 | 3.7 | 5.17 | 0.0230 |
| L1259 | <i>S. Typhimurium</i> SL1344 rif ^R pCT4 | 3.9 | 27.9 | <0.001 |
| L1260 | <i>S. Typhimurium</i> SL1344 rif ^R pCT5 | 3.6 | 8.45 | 0.0037 |
| I855 | <i>E. coli</i> 3950 pCT | 4.75 | | |
| I823 | <i>E. coli</i> 3950 | 4.75 | 0.019 | 0.8915 |
| I837 | <i>E. coli</i> 3950 pCT3 | 4.7 | 0.29 | 0.5902 |
| I831 | <i>E. coli</i> 3950 pCT4 | 4.8 | 0.14 | 0.7083 |
| I841 | <i>E. coli</i> 3950 pCT5 | 5.25 | 0.70 | 0.4028 |

Survival curves were constructed using a Kaplan Mier calculation showing the probability of *C. elegans* survival each day when fed continuously on the tested bacterial strains (Figure 6.6, page 223).

TD₅₀ = time taken for 50% of the nematodes to die,

Significance with respect to SL1344 rif^R pCT is denoted with bold text.

Appendix 34. Competition Index of plasmids pCT3-9 when compared *in vitro* against wild-type pCT

| | pCT2 | pCT3 | pCT4 | pCT5 | pCT6 | pCT7 | pCT8 | pCT9 |
|----------------------|---------|--------|--------|--------|--------|--------|--------|--------|
| % at 72 hours | 45% | 53% | 52% | 51% | 43% | 46% | 51% | 47% |
| SD | 8.2% | 3.0% | 8.5% | 8.4% | 6.2% | 3.8% | 1.2% | 8.3% |
| CI | 0.99985 | 1.0006 | 1.0012 | 1.0006 | 0.9985 | 0.9992 | 1.0002 | 0.9996 |

Each pair-wise competition was conducted between *E. coli* DH5 α pCT and DH5 α containing the test plasmid *in vitro*. CI, competition index was calculated based on the mean values of at least three separate experiments.

Appendix 35. Biofilm formation of strains containing pCT and pCT 2-6

| Strain | C159/11 | 3950 | 3950 pCT | 3950 pCT2 | 3950 pCT2b | 3950 pCT3 | 3950 pCT4 | 3950 pCT5 | 3950 pCT6 |
|--------------------------------------|---------|--------|----------|-----------|------------|-----------|-----------|------------------|------------------|
| Optical density (OD ₆₀₀) | 0.0462 | 0.0476 | 0.0339 | 0.0473 | 0.1492 | 0.1188 | 0.1416 | 0.3635 | 0.0795 |
| SD | 0.0756 | 0.0379 | 0.0223 | 0.0221 | 0.3331 | 0.2930 | 0.2981 | 0.0812 | 0.0464 |
| P value (compared to 3950 pCT) | 0.55 | 0.22 | - | 0.079 | 0.152 | 0.228 | 0.120 | <0.001 | <0.001 |

| | 14028S | DH5α | DH5α pCT | DH5α pCT2 | DH5α pCT2b | DH5α pCT3 | DH5α pCT4 | DH5α pCT5 | DH5α pCT6 | DH5α pCT7 |
|--------------------------------------|--------|--------|----------|-----------|------------------|---------------|-----------|-----------|-----------|-----------|
| Optical density (OD ₆₀₀) | 1.7855 | 1.2328 | 1.0247 | 1.2041 | 0.1370 | 1.2607 | 1.0915 | 0.9151 | 1.2690 | 1.2432 |
| SD | 0.3972 | 0.2977 | 0.3678 | 0.7120 | 0.1400 | 0.2506 | 0.2601 | 0.3479 | 0.2494 | 0.2571 |
| P value (compared to DH5α pCT) | - | 0.07 | - | 0.35 | <0.001 | 0.03 | 0.53 | 0.37 | 0.07 | 0.06 |

The amount of biofilm each bacterial strain produced was measured by staining each biofilm with crystal violet and using optical density (OD₆₀₀) to quantify each biofilm. Optical density values of four technical repeats and at least four biological repeats were compared using a student T test. Significant values compared to strains containing plasmidpCT are denoted with bold text.

Appendix 36. The aggregative ability of *S. Typhimurium* SL1344 rif^R and *E. coli* J53-2 +/- pCT and the pCT mutants

A) *S. Typhimurium* SL1344 rif^R and *E. coli* J53-2 +/- pCT

| Lab no. | Strain | Percentage of the start optical density Time (hours) | | | | | Student's T-test (<i>P</i> value) |
|---------|-----------------------------|---|------|-----|-----|-----|--|
| | | 0 | 1 | 6 | 12 | 24 | |
| I845 | <i>E. coli</i> 042 | 100% | 68% | 58% | 32% | 21% | 0.17 |
| L1079 | SL1344 rif ^R pCT | 100% | 89% | 80% | 72% | 67% | |
| L354 | SL1344 rif ^R | 100% | 99% | 88% | 74% | 39% | |
| I834 | J53-2 pCT | 100% | 104% | 81% | 72% | 47% | 0.14 |
| I847 | J53-2 | 100% | 90% | 45% | 27% | 8% | |

Data corresponding to Figure 6.12 (page 233)

B) *S. Typhimurium* SL1344 rif^R and *E. coli* J53-2 +/- pCT , pCT4 and pCT5

| Lab no. | Strain | Percentage of the start optical density Time (hours) | | | | | Student's T-test (<i>P</i> value) |
|---------|------------------------------|---|------|-----|-----|-----|--|
| | | 0 | 1 | 6 | 12 | 24 | |
| I845 | <i>E. coli</i> 042 | 100% | 68% | 58% | 32% | 21% | 0.62 |
| L1079 | SL1344 rif ^R pCT | 100% | 89% | 80% | 72% | 67% | |
| L1259 | SL1344 rif ^R pCT4 | 100% | 102% | 72% | 73% | 31% | |
| L1260 | SL1344 rif ^R pCT5 | 100% | 95% | 76% | 73% | 51% | 0.92 |
| I834 | J53-2 pCT | 100% | 104% | 81% | 72% | 47% | 0.66 |
| I859 | J53-2 pCT4 | 100% | 91% | 63% | 73% | 46% | |
| I860 | J53-2 pCT5 | 100% | 82% | 72% | 73% | 46% | 0.89 |

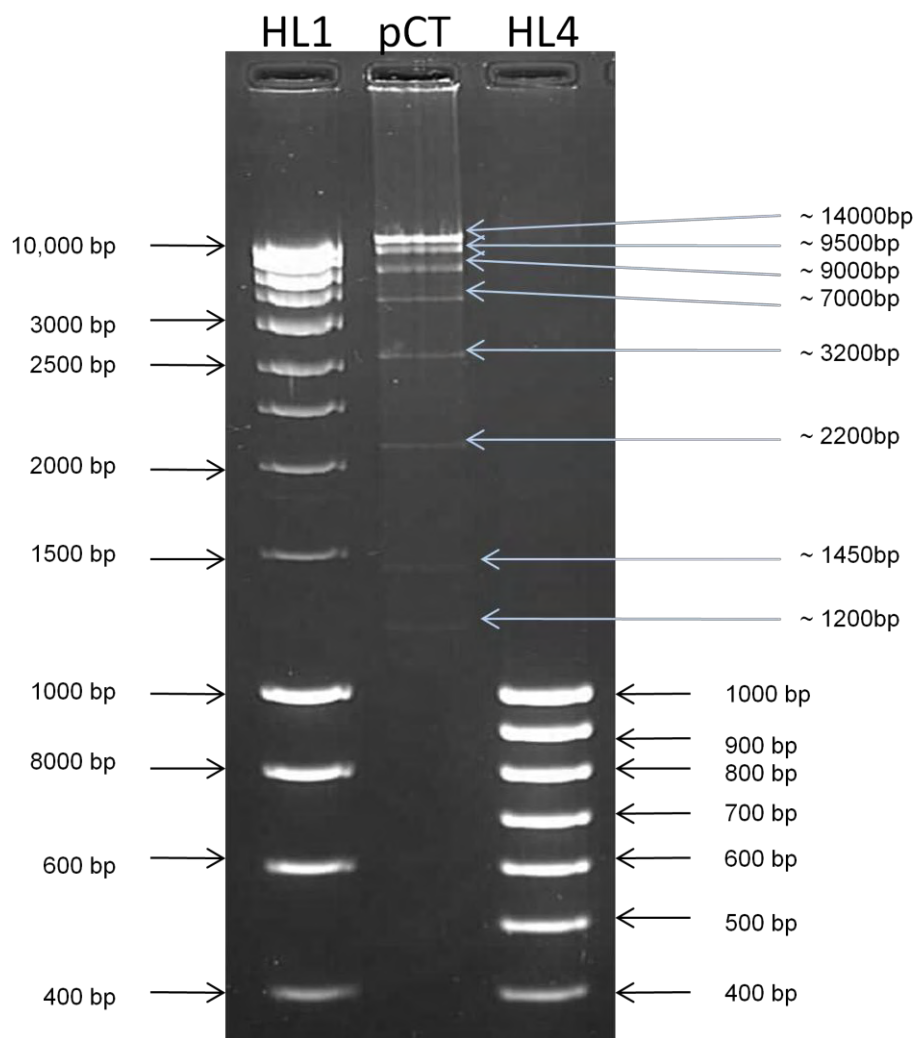
Data corresponding to Figure 6.13 (page 234)

C) *S. Typhimurium* SL1344 rif^R and *E. coli* J53-2 +/- pCT2, pCT2b, pCT3 and pCT6

| Lab no. | Strain | Percentage of the start optical density Time (hours) | | | | | Student's T-test (<i>P</i> value) |
|---------|-------------------------------|---|------|-----|-----|-----|--|
| | | 0 | 1 | 6 | 12 | 24 | |
| I845 | <i>E. coli</i> 042 | 100% | 68% | 58% | 32% | 21% | 0.06 |
| L1079 | SL1344 rif ^R pCT | 100% | 89% | 80% | 72% | 67% | |
| L1244 | SL1344 rif ^R pCT2 | 100% | 80% | 87% | 59% | 36% | |
| L1235 | SL1344 rif ^R pCT2b | 100% | 89% | 79% | 71% | 36% | 0.31 |
| L1236 | SL1344 rif ^R pCT3 | 100% | 96% | 82% | 78% | 37% | 0.90 |
| L1245 | SL1344 rif ^R pCT6 | 100% | 101% | 76% | 76% | 46% | 0.95 |
| I855 | J53-2 pCT | 100% | 104% | 81% | 72% | 47% | 0.67 |
| I856 | J53-2 pCT2 | 100% | 101% | 67% | 35% | 7% | |
| I857 | J53-2 pCT2b | 100% | 97% | 74% | 49% | 17% | 0.87 |
| I858 | J53-2 pCT3 | 100% | 114% | 78% | 44% | 18% | 0.48 |
| I861 | J53-2 pCT6 | 100% | 112% | 73% | 39% | 21% | 0.88 |

Data corresponding to Figure 6.14 (page 235)

Appendix 37. Restriction map of plasmid pCT digested with *Eco*R1 on a 1.2% agarose gel



HL1 = Hyperladder 1 sizing marker
HL4 = Hyperladder 4 sizing marker

References

- Aarestrup, F.M., (2004) Monitoring of antimicrobial resistance among food animals: principles and limitations. *Journal of Veterinary Medicine B Infectious Diseases and Veterinary Public Health*. **51** (8-9) 380-388.
- Aballay, A. and F.M. Ausubel, (2001) Programmed cell death mediated by *ced-3* and *ced-4* protects *Caenorhabditis elegans* from *Salmonella* Typhimurium-mediated killing. *Proceedings of the National Academy of Sciences of the USA*. **98** (5) 2735-2739.
- Aballay, A., P. Yorgey and F.M. Ausubel, (2000) *Salmonella* Typhimurium proliferates and establishes a persistent infection in the intestine of *Caenorhabditis elegans*. *Current Biology*. **10** (23) 1539-1542.
- Abraham, E.P., (1987) Cephalosporins 1945-1986. *Drugs*. **34** Suppl 2, 1-14.
- Abraham, E.P. and E. Chain. (1940) An enzyme from bacteria able to destroy penicillin. *Nature* **37** (13) 837; A Centenary Perspective, edited by Wolfgang K. Joklik, ASM Press. (1999) p.1115.
- Abromaitis, S., S. Faucher, M. Béland, R. Curtiss and F. Daigle, (2005) The presence of the *tet* gene from cloning vectors impairs *Salmonella* survival in macrophages. *FEMS Microbiology Letters*. **242** (2) 305-312.
- Alobwede, I., F.H. M'Zali, D.M. Livermore, J. Heritage, N. Todd and P.M. Hawkey, (2003) CTX-M extended-spectrum beta-lactamase arrives in the UK. *Journal of Antimicrobial Chemotherapy*. **51** (2) 470-471.
- Alonso, A., P. Sanchez and J.L. Martinez, (2001) Environmental selection of antibiotic resistance genes. *Environmental Microbiology*. **3** (1) 1-9.
- Amabile-Cuevas, C.F. and J.A. Heinemann, (2004) Shooting the messenger of antibiotic resistance: Plasmid elimination as a potential counter-evolutionary tactic. *Drug Discovery Today*. **9** (11) 465-467.
- Ambler, R.P., A.F. Coulson, J.M. Frere, J.M. Ghuysen, B. Joris, M. Forsman, R.C. Levesque, G. Tiraby and S.G. Waley, (1991) A standard numbering scheme for the class A beta-lactamases. *The Biochemical Journal*. **276** (Pt 1) 269-270.
- Anderson, J.D., (1974) The Effect of R-Factor Carriage on the Survival of *Escherichia coli* in The Human Intestine. *Journal of Medical Microbiology*. **7** (1) 85-90.
- Andersson, D.I., (2006) The biological cost of mutational antibiotic resistance: any practical conclusions? *Current Opinion in Microbiology*. **9** (5) 461-465.
- Andersson, D.I. and D. Hughes, (2010) Antibiotic resistance and its cost: is it possible to reverse resistance? *Nature Reviews Microbiology*. **8** (4) 260-271.
- Andersson, D.I. and B.R. Levin, (1999) The biological cost of antibiotic resistance. *Current Opinion in Microbiology*. **2** (5) 489-493.
- Andrews, J.M., (2011) "BSAC Methods for Antimicrobial Susceptibility Testing". (Accessed on May 2011).

- Antao, E.-M., L. Wieler and C. Ewers, (2009) Adhesive threads of extraintestinal pathogenic *Escherichia coli*. *Gut Pathogens*. **1** (1) 22.
- Arango Pinedo, C. and B.F. Smets, (2005) Conjugal TOL Transfer from *Pseudomonas putida* to *Pseudomonas aeruginosa*: Effects of Restriction Proficiency, Toxicant Exposure, Cell Density Ratios, and Conjugation Detection Method on Observed Transfer Efficiencies. *Applied Environmental Microbiology*. **71** (1) 51-57.
- Asai, K., K. Ishiwata, K. Matsuzaki and Y. Sadaie, (2008) A viable *Bacillus subtilis* strain without functional extracytoplasmic function sigma genes. *Journal of Bacteriology*. **190** (7) 2633-2636.
- Asano, K., C. Hama, S. Inoue, H. Moriwaki and K. Mizobuchi (1999) The plasmid Collb-P9 antisense Inc RNA controls expression of the RepZ replication protein and its positive regulator *repY* with different mechanisms. *Journal of Biological Chemistry*. **274** (25) 17924-17933.
- Bae, I.K., Y.H. Lee, H.J. Jeong, S.G. Hong, S.H. Lee and S.H. Jeong, (2008) A novel *bla*_{CTX-M-14} gene-harboring complex class 1 integron with an In4-like backbone structure from a clinical isolate of *Escherichia coli*. *Diagnostic Microbiology and Infectious Disease*. **62** (3) 340-342.
- Bahl, M.I., L.H. Hansen, T.R. Licht and S.J. Sorensen, (2007) Conjugative transfer facilitates stable maintenance of IncP-1 plasmid pJK5 in *Escherichia coli* cells colonizing the gastrointestinal tract of the germfree rat. *Applied Environmental Microbiology*. **73** (1) 341-343.
- Bailey, A.M., A. Ivens, R. Kingsley, J.L. Cottell, J. Wain and L.J.V. Piddock, (2010) RamA, a Member of the AraC/XylS Family, Influences Both Virulence and Efflux in *Salmonella enterica* Serovar Typhimurium. *Journal of Bacteriology*. **192** (6) 1607-1616.
- Bardhan, P., Faruque, A.S.G., Naheed, A and Sack, D.A. (2010) Decreasing Shigellosis-related deaths without *Shigella* spp.-specific interventions, Asia. *Emerging Infectious Diseases*, **16** (11) 1718-23
- Barr, V., K. Barr, M.R. Millar and R.W. Lacey, (1986) β -lactam antibiotics increase the frequency of plasmid transfer in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy*. **17** (4) 409-413.
- Barthelemy, M., J. Peduzzi, H. Bernard, C. Tancrede and R. Labia, (1992) Close amino acid sequence relationship between the new plasmid-mediated extended-spectrum beta-lactamase MEN-1 and chromosomally encoded enzymes of *Klebsiella oxytoca*. *Biochimica et Biophysica Acta*. **1122** (1) 15-22.
- Batchelor, M., K. Hopkins, E.J. Threlfall, F.A. Clifton-Hadley, A.D. Stallwood, R.H. Davies and E. Liebana, (2005) *bla*_{CTX-M} genes in clinical *Salmonella* isolates recovered from humans in England and Wales from 1992 to 2003. *Antimicrobial Agents and Chemotherapy*. **49** (4) 1319-1322.

- Baudry, P.J., L. Mataseje, G.G. Zhanel, D.J. Hoban and M.R. Mulvey, (2009) Characterization of plasmids encoding CMY-2 AmpC beta-lactamases from *Escherichia coli* in Canadian intensive care units. *Diagnostic Microbiology and Infectious Disease*. **65** (4):379-83.
- Bauernfeind, A., J.M. Casellas, M. Goldberg, M. Holley, R. Jungwirth, P. Mangold, T. Rohnisch, S. Schweighart and R. Wilhelm, (1992) A new plasmidic cefotaximase from patients infected with *Salmonella* Typhimurium. *Infection*. **20** (3) 158-163.
- Bayer, M.E. and K. Easterbrook, (1991) Tubular spinae are long-distance connectors between bacteria. *Journal of General Microbiology*. **137** (5) 1081-1086.
- Bean, D.C., D.M. Livermore and L.M. Hall, (2009). Plasmids imparting sulfonamide resistance in *Escherichia coli*: implications for persistence. *Antimicrobial Agents and Chemotherapy*. **53** (3) 1088-1093.
- Bentley, W.E., N. Mirjalili, D.C. Andersen, R.H. Davis and D.S. Kompala, (1990). Plasmid-encoded protein: The principal factor in the "metabolic burden" associated with recombinant bacteria. *Biotechnology and Bioengineering*. **35** (7) 668-681.
- Berger, C.N., S.V. Sodha, R.K. Shaw, P.M. Griffin, D. Pink, P. Hand and G. Frankel, (2010) Fresh fruit and vegetables as vehicles for the transmission of human pathogens. *Environmental Microbiology*. **12** (9) 2385-2397.
- Billard-Pomares, T., O. Tenaillon, H. Le Nagard, Z. Rouy, S. Cruveiller, C. Medigue, G. Arlet, E. Denamur and C. Branger, (2011) Complete nucleotide sequence of plasmid pTN48 encoding the CTX-M-14 extended spectrum beta-lactamase from an *Escherichia coli* O102-ST405 strain. *Antimicrobial Agents and Chemotherapy*. **55** (3):1270-3.
- Birnboim, H.C. and J. Doly, (1979) A rapid alkaline extraction procedure for screening recombinant plasmid DNA. *Nucleic Acids Res*. **7** (6) 1513-1523.
- Bishop, R.E. and J.H. Weiner, (1992) Coordinate regulation of murein peptidase activity and AmpC beta-lactamase synthesis in *Escherichia coli*. *FEBS Lett*. **304** (2-3) 103-108.
- Bjorkholm, B., M. Sjolund, P.G. Falk, O.G. Berg, L. Engstrand and D.I. Andersson, (2001) Mutation frequency and biological cost of antibiotic resistance in *Helicobacter pylori*. *Proceedings of the National Academy of Sciences of the United States of America*. **98** (25) 14607-14612.
- Björkman, J. and D.I. Andersson, (2000) The cost of antibiotic resistance from a bacterial perspective. *Drug Resistance Updates*. **3** (4) 237-245.
- Bjorkman, J., D. Hughes and D.I. Andersson, (1998) Virulence of antibiotic-resistant *Salmonella* Typhimurium. *Proceedings of the National Academy of Sciences of the United States of America*. **95** (7) 3949-3953.
- Black, R.E., (1993) Epidemiology of diarrhoeal disease: implications for control by vaccines. *Vaccine*. **11** (2) 100-106.

- Blair, J.M.A., R.M. La Ragione, M.J. Woodward and L.J.V. Piddock, (2009) Periplasmic adaptor protein AcrA has a distinct role in the antibiotic resistance and virulence of *Salmonella enterica* serovar Typhimurium. *Journal of Antimicrobial Chemotherapy*. **64** (5) 965-972.
- Blanc, V., P. Cortes, R.J. Mesa, E. Miro, F. Navarro and M. Llagostera, (2008) Characterisation of plasmids encoding extended-spectrum beta-lactamase and CMY-2 in *Escherichia coli* isolated from animal farms. *International Journal of Antimicrobial Agents* **31** (1) 76-78.
- Blanc, V., R. Mesa, M. Saco, S. Lavilla, G. Prats, E. Miro, F. Navarro, P. Cortes and M. Llagostera, (2006) ESBL and plasmidic class C beta-lactamase-producing *E. coli* strains isolated from poultry, pig and rabbit farms. *Veterinary Microbiology*. **118** (3-4) 299-304.
- Blanco, M., M.P. Alonso, M.H. Nicolas-Chanoine, G. Dahbi, A. Mora, J.E. Blanco, C. Lopez, P. Cortes, M. Llagostera, V. Leflon-Guibout, B. Puentes, R. Mamani, A. Herrera, M.A. Coira, F. Garcia-Garrote, J.M. Pita and J. Blanco, (2009) Molecular epidemiology of *Escherichia coli* producing extended-spectrum beta-lactamases in Lugo (Spain): dissemination of clone O25b:H4-ST131 producing CTX-M-15. *Journal of Antimicrobial Chemotherapy*. **63** (6):1135-41
- BNF60, Ed. 60, (2011) British National Formulary 60. London, UK, RPS publishing. Section 5, page 283
- Bonnet, R., (2004) Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrobial Agents and Chemotherapy*. **48** (1) 1-14.
- Bonnet, R., C. Dutour, J.L. Sampaio, C. Chanal, D. Sirot, R. Labia, C. De Champs and J. Sirot, (2001) Novel cefotaximase (CTX-M-16) with increased catalytic efficiency due to substitution Asp-240-->Gly. *Antimicrobial Agents and Chemotherapy*. **45** (8) 2269-2275.
- Bortolaia, V., L. Guardabassi, M. Bisgaard, J. Larsen and A.M. Bojesen, (2010) *Escherichia coli* Producing CTX-M-1, -2, and -9 Group beta-Lactamases in Organic Chicken Egg Production. *Antimicrobial Agents and Chemotherapy*. **54** (8) 3527-3528.
- Bou, G., M. Cartelle, M. Tomas, D. Canle, F. Molina, R. Moure, J.M. Eiros and A. Guerrero, (2002) Identification and broad dissemination of the CTX-M-14 beta-lactamase in different *Escherichia coli* strains in the northwest area of Spain. *Journal of Clinical Microbiology*. **40** (11) 4030-4036.
- Bouma, J.E. and R.E. Lenski, (1988). Evolution of a bacteria/plasmid association. *Nature*. **335** (6188) 351-352.
- Bradford, P.A., (2001) Extended-spectrum beta-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clinical Microbiology Reviews*. **14** (4) 933-951.
- Bradley, D.E., (1980) Morphological and serological relationships of conjugative pili. *Plasmid*. **4** (2) 155-169.
- Bradley, D.E., (1984). Characteristics and function of thick and thin conjugative pili determined by transfer-derepressed plasmids of incompatibility groups I1, I2, I5, B, K and Z. *Journal of General Microbiology*. **130** (6) 1489-1502.

- Brenwald, N.P., G. Jevons, J.M. Andrews, J.H. Xiong, P.M. Hawkey and R. Wise, (2003). An outbreak of a CTX-M-type beta-lactamase-producing *Klebsiella pneumoniae*: the importance of using cefpodoxime to detect extended-spectrum beta-lactamases. *Journal of Antimicrobial Chemotherapy*. **51** (1) 195-196.
- Brinas, L., M.A. Moreno, M. Zarazaga, C. Porrero, Y. Saenz, M. Garcia, L. Dominguez and C. Torres, (2003). Detection of CMY-2, CTX-M-14, and SHV-12 beta-lactamases in *Escherichia coli* fecal-sample isolates from healthy chickens. *Antimicrobial Agents and Chemotherapy*. **47** (6) 2056-2058.
- Brussow, H., C. Canchaya and W.D. Hardt, (2004). Phages and the evolution of bacterial pathogens: from genomic rearrangements to lysogenic conversion. *Microbiology and Molecular Biology Reviews*. **68** (3) 560-602.
- Buckley, A.M., M.A. Webber, S. Cooles, L.P. Randall, R.M. La Ragione, M.J. Woodward and L.J. Piddock, (2006). The AcrAB-TolC efflux system of *Salmonella enterica* serovar Typhimurium plays a role in pathogenesis. *Cellular Microbiology*. **8** (5) 847-856.
- Bush, K., (2010). Alarming beta-lactamase-mediated resistance in multidrug-resistant *Enterobacteriaceae*. *Current Opinion in Microbiology*. **13** (5) 558-564.
- Bush, K. and J.F. Fisher, (2011). Epidemiological Expansion, Structural Studies and Clinical Challenges of New beta-Lactamases from Gram-Negative Bacteria. *Annual Review of Microbiology*. **65**, 455-478.
- Bush, K. and G.A. Jacoby, (2010). Updated Functional Classification of beta-Lactamases. *Antimicrobial Agents and Chemotherapy*. **54** (3) 969-976.
- Bush, K., G.A. Jacoby and A.A. Medeiros, (1995). A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrobial Agents and Chemotherapy*. **39** (6) 1211-1233.
- Bush, L.M., J. Calmon and C.C. Johnson, (1995). Newer penicillins and beta-lactamase inhibitors. *Infectious Disease Clinics of North America*. **9** (3) 653-686.
- Call, D.R., R.S. Singer, D. Meng, S.L. Broschat, L.H. Orfe, J.M. Anderson, D.R. Herndon, L.S. Kappmeyer, J.B. Daniels and T.E. Besser, (2009). *bla*_{CMY-2} positive IncA/C plasmids from *Escherichia coli* and *Salmonella enterica* are a distinct component of a larger lineage of plasmids. *Antimicrobial Agents and Chemotherapy*. **54** (2):590-6.
- Cantón, R. and T.M. Coque, (2006). The CTX-M beta-lactamase pandemic. *Current Opinion in Microbiology*. **9** (5) 466-475.
- Canton, R., A. Novais, A. Valverde, E. Machado, L. Peixe, F. Baquero and T.M. Coque, (2008) Prevalence and spread of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* in Europe. *Clinical Microbiology and Infection*. Suppl 1, 144-153.
- Cao, V., T. Lambert, D.Q. Nhu, H.K. Loan, N.K. Hoang, G. Arlet and P. Courvalin, (2002). Distribution of extended-spectrum beta-lactamases in clinical isolates of *Enterobacteriaceae* in Vietnam. *Antimicrobial Agents and Chemotherapy*. **46** (12) 3739-3743.

- Carattoli, A., (2008) Animal reservoirs for extended spectrum beta-lactamase producers. *Clinical Microbiology and Infection*. Suppl 1, 117-123.
- Carattoli, A., (2009) Resistance plasmid families in *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy*. **53** (6) 2227-2238.
- Carattoli, A., A. Bertini, L. Villa, V. Falbo, K.L. Hopkins and E.J. Threlfall, (2005). Identification of plasmids by PCR-based replicon typing. *Journal of Microbiological Methods*. **63** (3) 219-228.
- Carattoli, A., V. Miriagou, A. Bertini, A. Loli, C. Colinon, L. Villa, J.M. Whichard and G.M. Rossolini, (2006). Replicon typing of plasmids encoding resistance to newer beta-lactams. *Emerging Infectious Diseases*. **12** (7) 1145-1148.
- Chain, P.S.G., D.V. Grafham, R.S. Fulton, M.G. FitzGerald, J. Hostetler, D. Muzny, J. Ali, B. Birren, D.C. Bruce, C. Buhay, J.R. Cole, Y. Ding, S. Dugan, D. Field, G.M. Garrity, R. Gibbs, T. Graves, C.S. Han, S.H. Harrison, S. Highlander, P. Hugenholtz, H.M. Khouri, C.D. Kodira, E. Kolker, N.C. Kyrpides, D. Lang, A. Lapidus, S.A. Malfatti, V. Markowitz, T. Metha, K.E. Nelson, J. Parkhill, S. Pitluck, X. Qin, T.D. Read, J. Schmutz, S. Sozhamannan, P. Sterk, R.L. Strausberg, G. Sutton, N.R. Thomson, J.M. Tiedje, G. Weinstock, A. Wollam, C. Genomic Standards Consortium Human Microbiome Project Jumpstart and J.C. Detter, (2009) Genome Project Standards in a New Era of Sequencing. *Science*. **326** (5950) 236-237.
- Chanawong, A., F.H. M'Zali, J. Heritage, J.H. Xiong and P.M. Hawkey, (2002) Three cefotaximases, CTX-M-9, CTX-M-13, and CTX-M-14, among *Enterobacteriaceae* in the People's Republic of China. *Antimicrobial Agents and Chemotherapy*. **46** (3) 630-637.
- Chaslus-Dancla, E., G. Gerbaud, M. Lagorce, J.P. Lafont and P. Courvalin, (1987) Persistence of an antibiotic resistance plasmid in intestinal *Escherichia coli* of chickens in the absence of selective pressure. *Antimicrobial Agents and Chemotherapy*. **31** (5) 784-788.
- Chigor, V.N., Umoh, V.J., Smith, S.I., Igbinosa, E.O., and Okoh, A.I., (2010) Multidrug Resistance and Plasmid Patterns of *Escherichia coli* O157 and Other *E. coli* Isolated from Diarrhoeal Stools and Surface Waters from Some Selected Sources in Zaria, Nigeria. *International Journal of Environmental Research and Public Health*. **7** (10) 3831–3841.
- Clermont, O., H. Dhanji, M. Upton, T. Gibreel, A. Fox, D. Boyd, M.R. Mulvey, P. Nordmann, E. Ruppe, J.L. Sarthou, T. Frank, S. Vimont, G. Arlet, C. Branger, N. Woodford and E. Denamur, (2009) Rapid detection of the O25b-ST131 clone of *Escherichia coli* encompassing the CTX-M-15-producing strains. *Journal of Antimicrobial Chemotherapy*. **64** (2) 274-277.
- Clowes, R.C., (1972) Molecular structure of bacterial plasmids. *Bacteriological Reviews*. **36** (3) 361-405.
- Clowes, R.C. and D. Rowley, (1954) Some Observations on Linkage Effects in Genetic Recombination in *Escherichia coli* K-12. *Journal of General Microbiology*. **11** (2) 250-260.
- Coetzee, J.N., D.E. Bradley and R.W. Hedges, (1982) Phages I α and I2-2: Incl Plasmid-dependent Bacteriophages. *Journal of General Microbiology*. **128** (11) 2797-2804.

- Coetzee, J.N., F.A. Sirgel and G. Lecatsas, (1980) Properties of a Filamentous Phage which Adsorbs to Pili Coded by Plasmids of the IncI Complex. *Journal of General Microbiology*. **117** (2) 547-551.
- Coldham, N.G., M. Stokes, J.L. Cottell, L. Randall, H. Wearing, L. Snow, M. Wootton, R. Howe, L.J.V. Piddock and C. Teale, (2011) Molecular epidemiology of CTX-M-14 ESBL *E. coli* from cattle and humans in England and Wales. 21st European Congress of Clinical Microbiology and Infectious Diseases. R2348.
- Coque, T.M., A. Novais, A. Carattoli, L. Poirel, J. Pitout, L. Peixe, F. Baquero, R. Canton and P. Nordmann, (2008) Dissemination of clonally related *Escherichia coli* strains expressing extended-spectrum beta-lactamase CTX-M-15. *Emerging Infectious Disease*. **14** (2) 195-200.
- Coronado, C., M.E. Vázquez, A. Cebolla and A.J. Palomares, (1994) Use of Firefly Luciferase Gene for Plasmid Copy Number Determination. *Plasmid*. **32** (3) 336-341.
- Costa, D., P. Poeta, Y. Saenz, L. Vinue, A.C. Coelho, M. Matos, B. Rojo-Bezares, J. Rodrigues and C. Torres, (2008) Mechanisms of antibiotic resistance in *Escherichia coli* isolates recovered from wild animals. *Microbial Drug Resistance*. **14** (1) 71-77.
- Costa, D., P. Poeta, Y. Saenz, L. Vinue, B. Rojo-Bezares, A. Jouini, M. Zarazaga, J. Rodrigues and C. Torres, (2006) Detection of *Escherichia coli* harbouring extended-spectrum beta-lactamases of the CTX-M, TEM and SHV classes in faecal samples of wild animals in Portugal. *Journal of Antimicrobial Chemotherapy*. **58** (6) 1311-1312.
- Cottell, J.L., M.A. Webber, N.G. Coldham, D. Taylor, A.M. Cerdño-Tárraga, H. Hauser, N.R. Thomson, M.J. Woodward and L.J.V. Piddock, (2011) Complete Sequence and Molecular Epidemiology of an IncK Epidemic Plasmid Encoding *bla*_{CTX-M-14}. *Emerging Infectious Diseases*. **17** (4) 645-652.
- Couturier, M., F. Bex, P.L. Bergquist and W.K. Maas, (1988) Identification and classification of bacterial plasmids. *Microbiology Reviews*. **52** (3) 375-395.
- Crump, J.A., S.P. Luby and E.D. Mintz, (2004) The global burden of typhoid fever. *Bulletin of the World Health Organisation*. **82** (5) 346-353.
- Cullik, A., Y. Pfeifer, R. Prager, H. von Baum and W. Witte, (2010) A novel IS26 structure is surrounding *bla*_{CTX-M} genes in different plasmids of German clinical isolates of *Escherichia coli*. *Journal of Medical Microbiology*. **59** (Pt 5):580-7
- Cundliffe, E., (1989) How antibiotic-producing organisms avoid suicide. *Annual Review of Microbiology*. **43**, 207-233.
- Dahlberg, C., M. Bergstrom and M. Hermansson, (1998). *In-Situ* Detection of High Levels of Horizontal Plasmid Transfer in Marine Bacterial Communities. *Applied and Environmental Microbiology*. **64** (7) 2670 - 2675.
- Dahlberg, C. and L. Chao, (2003). Amelioration of the cost of conjugative plasmid carriage in *Escherichia coli* K12. *Genetics*. **165** (4) 1641-1649.

- Daniels, J.B., D.R. Call, D. Hancock, W.M. Sischo, K. Baker and T.E. Besser, (2009). Role of Ceftiofur in Selection and Dissemination of *bla*_{CMY-2}-Mediated Cephalosporin Resistance in *Salmonella enterica* and Commensal *Escherichia coli* Isolates from Cattle. *Applied Environmental Microbiology*. **75** (11) 3648-3655.
- Datsenko, K.A. and B.L. Wanner, (2000). One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proceedings of the National Academy of Sciences of the United States of America*. **97** (12) 6640-6645.
- Datta, N. and V.M. Hughes, (1983). Plasmids of the same Inc groups in *Enterobacteria* before and after the medical use of antibiotics. *Nature*. **306** (5943) 616-617.
- Davies, J. and D. Davies, (2010). Origins and Evolution of Antibiotic Resistance. *Microbiology and Molecular Biology Reviews*. **74** (3) 417-433.
- De Gelder, L., J.M. Ponciano, P. Joyce and E.M. Top, (2007). Stability of a promiscuous plasmid in different hosts: no guarantee for a long-term relationship. *Microbiology*. **153** (Pt 2) 452-463.
- DEFRA, (2007) Overview of Antimicrobial Usage and Bacterial Resistance in Selected Human and Animal Pathogens in the UK: 2007 (Last accessed August 2011).
- DeNap, J.C.B., J.R. Thomas, D.J. Musk and P.J. Hergenrother, (2004) Combating drug-resistant bacteria: small molecule mimics of plasmid incompatibility as anti-plasmid compounds. *Journal of American Chemical Society*. **126** (47) 15402-15404.
- Dhanji, H., M. Doumith, P.J. Rooney, M.C. O'Leary, A.C. Loughrey, R. Hope, N. Woodford and D.M. Livermore, (2010) Molecular epidemiology of fluoroquinolone-resistant ST131 *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in nursing homes in Belfast, UK. *Journal of Antimicrobial Chemotherapy*. **66** (2) 297-303.
- Dhanji, H., N.M. Murphy, C. Akhigbe, M. Doumith, R. Hope, D.M. Livermore and N. Woodford, (2010) Isolation of fluoroquinolone-resistant O25b:H4-ST131 *Escherichia coli* with CTX-M-14 extended-spectrum β -lactamase from UK river water. *Journal of Antimicrobial Chemotherapy*. **66** (3):512-6.
- Dibb-Fuller, M.P., E. Allen-Vercos, C.J. Thorns and M.J. Woodward, (1999) Fimbriae- and flagella-mediated association with and invasion of cultured epithelial cells by *Salmonella enteritidis*. *Microbiology*. **145** (5) 1023-1031.
- Dierikx, C., A. van Essen-Zandbergen, K. Veldman, H. Smith and D. Mevius, (2010) Increased detection of extended spectrum beta-lactamase producing *Salmonella enterica* and *Escherichia coli* isolates from poultry. *Veterinary Microbiology*. **145** (3-4) 273-8.
- Diestra, K., C. Juan, T. Curiao, B. Moya, E. Miro, J. Oteo, T.M. Coque, M. Perez-Vazquez, J. Campos, R. Canton, A. Oliver and F. Navarro, (2008) Characterization of plasmids encoding *bla*_{ESBL} and surrounding genes in Spanish clinical isolates of *Escherichia coli* and *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy*. **63** (1) 60-6.
- Dionisio, F., I.C. Conceicao, A.C. Marques, L. Fernandes and I. Gordo, (2005) The evolution of a conjugative plasmid and its ability to increase bacterial fitness. *Biology Letters*. **1** (2) 250-252.

- Dionisio, F., I. Matic, M. Radman, O.R. Rodrigues and F. Taddei, (2002) Plasmids spread very fast in heterogeneous bacterial communities. *Genetics*. **162** (4) 1525 - 1532.
- Dubois, D., N.V. Prasad Rao, R. Mittal, L. Bret, M. Roujou-Gris and R. Bonnet, (2009) CTX-M beta-lactamase production and virulence of *Escherichia coli* K1. *Emerging Infectious Diseases*. **15** (12) 1988-1990.
- Dudley, E.G., C. Abe, J.M. Ghigo, P. Latour-Lambert, J.C. Hormazabal and J.P. Nataro, (2006) An IncI1 plasmid contributes to the adherence of the atypical enteroaggregative *Escherichia coli* strain C1096 to cultured cells and abiotic surfaces. *Infection and Immunity*. **74** (4) 2102-2114.
- Duodu, S., G. Carlsson, K. Huss-Danell and M.M. Svenning, (2007) Large genotypic variation but small variation in N₂ fixation among rhizobia nodulating red clover in soils of northern Scandinavia. *Journal of Applied Microbiology*. **102** (6) 1625-1635.
- Dutour, C., R. Bonnet, H. Marchandin, M. Boyer, C. Chanal, D. Sirot and J. Sirot, (2002) CTX-M-1, CTX-M-3, and CTX-M-14 beta-lactamases from *Enterobacteriaceae* isolated in France. *Antimicrobial Agents and Chemotherapy*. **46** (2) 534-537.
- Eckert, C., V. Gautier and G. Arlet, (2006) DNA sequence analysis of the genetic environment of various *bla*_{CTX-M} genes. *Journal of Antimicrobial Chemotherapy*. **57** (1) 14-23.
- Eckert, S.E., F. Dziva, R.R. Chaudhuri, G.C. Langridge, D.J. Turner, D.J. Pickard, D.J. Maskell, N.R. Thomson and M.P. Stevens, (2011) Retrospective application of transposon-directed insertion site sequencing to a library of signature-tagged mini-Tn5Km2 mutants of *Escherichia coli* O157:H7 screened in cattle. *Journal of Bacteriology*. **193** (7) 1771-1776.
- Enne, V.I. and P.M. Bennett, (2006) The fitness cost imposed by plasmid RP1 on *Escherichia coli* differs from strain to strain. *Clinical Microbiology and Infection*. **12** (Suppl S4) P1234.
- Enne, V.I., P.M. Bennett, D.M. Livermore and L.M. Hall, (2004) Enhancement of host fitness by the *sul2*-coding plasmid p9123 in the absence of selective pressure. *Journal of Antimicrobial Chemotherapy*. **53** (6) 958-963.
- Enne, V.I., A.A. Delsol, G.R. Davis, S.L. Hayward, J.M. Roe and P.M. Bennett, (2005) Assessment of the fitness impacts on *Escherichia coli* of acquisition of antibiotic resistance genes encoded by different types of genetic element. *Journal of Antimicrobial Chemotherapy*. **56** (3) 544-551.
- Enne, V.I., D.M. Livermore, P. Stephens and L.M.C. Hall, (2001) Persistence of sulphonamide resistance in *Escherichia coli* in the UK despite national prescribing restriction. *The Lancet*. **357** (9265) 1325-1328.
- Ensor, V.M., M. Shahid, J.T. Evans and P.M. Hawkey, (2006). Occurrence, prevalence and genetic environment of CTX-M beta-lactamases in *Enterobacteriaceae* from Indian hospitals. *Journal of Antimicrobial Chemotherapy*. **58** (6) 1260-1263.
- Erickson, M.C. and M.P. Doyle, (2007) Food as a Vehicle for Transmission of Shiga Toxin Producing *Escherichia coli*. *Journal of Food Protection*. **70** (10) 2426-2449.

- Escudero, E., L. Vinue, T. Teshager, C. Torres and M.A. Moreno, (2009) Resistance mechanisms and farm-level distribution of faecal *Escherichia coli* isolates resistant to extended-spectrum cephalosporins in pigs in Spain. *Research in Veterinary Science*. **88** (1) 83-7.
- Ewers, C., M. Grobbel, I. Stamm, P.A. Kopp, I. Diehl, T. Semmler, A. Fruth, J. Beutlich, B. Guerra, L.H. Wieler and S. Guenther, (2010). Emergence of human pandemic O25:H4-ST131 CTX-M-15 extended-spectrum beta-lactamase-producing *Escherichia coli* among companion animals. *Journal of Antimicrobial Chemotherapy*. **65** (4) 651-60.
- Farmer, J.J., 3rd, G.R. Fanning, G.P. Huntley-Carter, B. Holmes, F.W. Hickman, C. Richard and D.J. Brenner, (1981) *Kluyvera*, a new (re-defined) genus in the family *Enterobacteriaceae*: identification of *Kluyvera ascorbata* sp. nov. and *Kluyvera cryocrescens* sp. nov. in clinical specimens. *Journal of Clinical Microbiology*. **13** (5) 919-933.
- Ferdy, J. and B. Godelle, (2005) Diversification of transmission modes and the evolution of mutualism. *The American naturalist*. **166** (5) 613-627.
- Fluit, A.C. and F.J. Schmitz, (2004) Resistance integrons and super-integrons. *Clinical Microbiology and Infection*. **10** (4) 272-288.
- Foucault, M.L., P. Courvalin and C. Grillot-Courvalin, (2009). Fitness cost of VanA-type vancomycin resistance in methicillin-resistant *Staphylococcus aureus*. *Antimicrobial Agents and Chemotherapy*. **53** (6) 2354-2359.
- Foxman, B., (2002) Epidemiology of urinary tract infections: incidence, morbidity, and economic costs. *The American Journal of Medicine*. **113** (Supplement 1) 5-13.
- Francia, M.V., A. Varsaki, M.P. Garcillán-Barcia, A. Latorre, C. Drainas and F. de la Cruz, (2004) A classification scheme for mobilization regions of bacterial plasmids. *FEMS Microbiology Reviews*. **28** (1) 79-100.
- Freitag, C.S., J.M. Abraham, J.R. Clements and B.I. Eisenstein, (1985). Genetic analysis of the phase variation control of expression of type 1 fimbriae in *Escherichia coli*. *Journal of Bacteriology*. **162** (2) 668-675.
- Friebs, K., 2004. Plasmid copy number and plasmid stability. *Advances in Biochemical Engineering and Biotechnology*. **86**, 47-82.
- Frost, L.S., K. Ippen-Ihler and R.A. Skurray, (1994) Analysis of the sequence and gene products of the transfer region of the F sex factor. *Microbiological Reviews*. **58** (2) 162-210.
- Furtado, G. and D. Nicolau, (2010) Overview perspective of bacterial resistance. *Expert Opinion on Therapeutic Patents*. **20** (10) 1273-1276.
- Furuya, N. and T. Komano, (1996) Nucleotide sequence and characterization of the *trbABC* region of the IncI1 Plasmid R64: existence of the *pnd* gene for plasmid maintenance within the transfer region. *Journal of Bacteriology*. **178** (6) 1491-1497.

- Garcia-Fernandez, A., G. Chiaretto, A. Bertini, L. Villa, D. Fortini, A. Ricci and A. Carattoli, (2008). Multilocus sequence typing of IncI1 plasmids carrying extended-spectrum beta-lactamases in *Escherichia coli* and *Salmonella* of human and animal origin. *Journal of Antimicrobial Chemotherapy*. **61** (6) 1229-1233.
- Garcillan-Barcia, M.P., M.V. Francia and F. de la Cruz, (2009). The diversity of conjugative relaxases and its application in plasmid classification. *FEMS Microbiology Reviews*. **33** (3) 657-687.
- Ghigo, J.M., (2001). Natural conjugative plasmids induce bacterial biofilm development. *Nature*. **412** (6845) 442-445.
- Gill, S.R., M. Pop, R.T. DeBoy, P.B. Eckburg, P.J. Turnbaugh, B.S. Samuel, J.I. Gordon, D.A. Relman, C.M. Fraser-Liggett and K.E. Nelson, (2006). Metagenomic Analysis of the Human Distal Gut Microbiome. *Science*. **312** (5778) 1355-1359.
- Gniadkowski, M., (2008). Evolution of extended-spectrum beta-lactamases by mutation. *Clinical Microbiology and Infection*. **14**, Suppl 1, 11-32.
- Gniadkowski, M., I. Schneider, A. Palucha, R. Jungwirth, B. Mikiewicz and A. Bauernfeind, (1998) Cefotaxime-resistant *Enterobacteriaceae* isolates from a hospital in Warsaw, Poland: identification of a new CTX-M-3 cefotaxime-hydrolyzing beta-lactamase that is closely related to the CTX-M-1/MEN-1 enzyme. *Antimicrobial Agents and Chemotherapy*. **42** (4) 827-832.
- Gonzalez-Sanz, R., S. Herrera-Leon, M. de la Fuente, M. Arroyo and M.A. Echeita, (2009) Emergence of extended-spectrum beta-lactamases and AmpC-type beta-lactamases in human *Salmonella* isolated in Spain from 2001 to 2005. *Journal of Antimicrobial Chemotherapy*. **64** (6) 1181-6.
- Goossens, H., M. Ferech, R. Vander Stichele and M. Elseviers, (2005). Outpatient antibiotic use in Europe and association with resistance: a cross-national database study. *The Lancet*. **365** (9459) 579-587.
- Gotz, A., R. Pukall, E. Smit, E. Tietze, R. Prager, H. Tschape, J.D. van Elsas and K. Smalla, (1996). Detection and characterization of broad-host-range plasmids in environmental bacteria by PCR. *Applied Environmental Microbiology*. **62** (7) 2621-2628.
- Grant, A.J., O. Restif, T.J. McKinley, M. Sheppard, D.J. Maskell and P. Mastroeni, (2008) Modelling within-host spatiotemporal dynamics of invasive bacterial disease. *PLoS Biology*. **6** (4) e74.
- Grindley, N.D., J.N. Grindley and E.S. Anderson, (1972) R factor compatibility groups. *Molecular and General Genetics*. **119** (4) 287-297.
- Gualdi, L., L. Tagliabue, S. Bertagnoli, T. Ierana, C. De Castro and P. Landini, (2008) Cellulose modulates biofilm formation by counteracting curli-mediated colonization of solid surfaces in *Escherichia coli*. *Microbiology*. **154** (7) 2017-2024.
- Guenther, S., M. Grobbel, J. Beutlich, B. Guerra, R.G. Ulrich, L.H. Wieler and C. Ewers, (2010) Detection of pandemic B2-O25-ST131 *Escherichia coli* harbouring the CTX-M-9 extended-spectrum beta-lactamase type in a feral urban brown rat (*Rattus norvegicus*). *Journal of Antimicrobial Chemotherapy*. **65** (3) 582-584.

- Guerrant, R.L., T. Van Gilder, T.S. Steiner, N.M. Thielman, L. Slutsker, R.V. Tauxe, T. Hennessy, P.M. Griffin, H. DuPont, R. Bradley Sack, P. Tarr, M. Neill, I. Nachamkin, L.B. Reller, M.T. Osterholm, M.L. Bennish and L.K. Pickering, (2001) Practice Guidelines for the Management of Infectious Diarrhea. *Clinical Infectious Diseases*. **32** (3) 331-351.
- Gyohda, A., N. Furuya, A. Ishiwa, S. Zhu and T. Komano, (2004) Structure and function of the shufflon in plasmid R64. *Advances in Biophysics*. **38**, 183-213.
- Haft, R.J., G. Palacios, T. Nguyen, M. Mally, E.G. Gachelet, E.L. Zechner and B. Traxler, (2006) General mutagenesis of F plasmid *Tral* reveals its role in conjugative regulation. *Journal of Bacteriology*. **188** (17) 6346-6353.
- Haft, R.J.F., J.E. Mittler and B. Traxler, (2009) Competition favours reduced cost of plasmids to host bacteria. *ISME Journal*. **3** (7) 761-769.
- Hale, L., O. Lazos, A. Haines and C. Thomas, (2010) An efficient stress-free strategy to displace stable bacterial plasmids. *Biotechniques*. **48** (3) 223-228.
- Hall-Stoodley, L., J.W. Costerton and P. Stoodley, (2004) Bacterial biofilms: from the Natural environment to infectious diseases. *Nature Reviews Microbiology*. **2** (2) 95-108.
- Hall, B.G. and M. Barlow, (2004) Evolution of the serine beta-lactamases: past, present and future. *Drug Resistance Updates*. **7** (2) 111-123.
- Han, C.G., Y. Shiga, T. Tobe, C. Sasakawa and E. Ohtsubo, (2001) Structural and functional characterization of IS679 and IS66-family elements. *Journal of Bacteriology*. **183** (14) 4296-4304.
- Hartley, C.L., K. Howe, A.H. Linton, K.B. Linton and M.H. Richmond, (1975) Distribution of R plasmids among the O-antigen types of *Escherichia coli* isolated from human and animal sources. *Antimicrobial Agents and Chemotherapy*. **8** (2) 122-131.
- Hartskeerl, R., E. Zuidweg, M. Van Geffen and W. Hoekstra, (1985) The IncI Plasmids R144, R64 and Collb Belong to One Exclusion Group. *Journal of General Microbiology*. **131** (6) 1305-1311.
- Hausner, M. and S. Wuertz, (1999) High rates of conjugation in bacterial biofilms as determined by quantitative *in-situ* analysis. *Applied and Environmental Microbiology*. **65** (8) 3710 - 3713.
- Hawkey, P.M., (2008) The growing burden of antimicrobial resistance. *Journal of Antimicrobial Chemotherapy*. **62** Suppl 1, i1-9.
- Hawkey, P.M., (2008) Molecular epidemiology of clinically significant antibiotic resistance genes. *British Journal of Pharmacology*. **153** Suppl 1, S406-413.
- Hawkey, P.M. and A.M. Jones, (2009) The changing epidemiology of resistance. *Journal of Antimicrobial Chemotherapy*. **64** Suppl 1, i3-10.
- Hayes, F., (2003) Toxins-antitoxins: plasmid maintenance, programmed cell death, and cell cycle arrest. *Science*. **301** (5639) 1496-1499.
- Hedges, R.W. and N. Datta, (1973) Plasmids determining I pili constitute a compatibility complex. *Journal of General Microbiology*. **77** (1) 19-25.

- Heuer, H., C. Kopmann, C.T. Binh, E.M. Top and K. Smalla, (2008) Spreading antibiotic resistance through spread manure: characteristics of a novel plasmid type with low %G+C content. *Environmental Microbiology*. **11** (4) 937-49.
- Hinton, M. and A.H. Linton, (1983) Antibacterial drug resistance among *Escherichia coli* isolated from calves fed on a milk substitute diet. *Veterinary Records*. **112** (24) 567-568.
- Hirsch, E.B. and V.H. Tam, (2011) Impact of multidrug-resistant *Pseudomonas aeruginosa* infection on patient outcomes. *Expert Review of Pharmacoeconomics & Outcomes Research*. **10** (4) 441-451.
- Ho, P.L., W.U. Lo, R.C.W. Wong, M.K. Yeung, K.H. Chow, T.L. Que, A.H.Y. Tong, J.Y.J. Bao, S. Lok and S.S.Y. Wong, (2011) Complete sequencing of the FII plasmid pHK01, encoding CTX-M-14, and molecular analysis of its variants among *Escherichia coli* from Hong Kong. *Journal of Antimicrobial Chemotherapy*. **66** (4) 752-756.
- Hohmann, E.L., (2001) Nontyphoidal salmonellosis. *Clinical Infectious Diseases*. **32** (2) 263-269.
- Hopkins, K.L., E. Liebana, L. Villa, M. Batchelor, E.J. Threlfall and A. Carattoli, (2006) Replicon typing of plasmids carrying CTX-M or CMY beta-lactamases circulating among *Salmonella* and *Escherichia coli* isolates. *Antimicrobial Agents and Chemotherapy*. **50** (9) 3203-3206.
- Hossain, A., M.D. Reisbig and N.D. Hanson, (2004) Plasmid-encoded functions compensate for the biological cost of AmpC overexpression in a clinical isolate of *Salmonella* Typhimurium. *Journal of Antimicrobial Chemotherapy*. **53** (6) 964-970.
- HPA (2011) "Escherichia coli bacteraemia in England, Wales and Northern Ireland 2006-2010; http://www.hpa.org.uk/web/HPAwebFile/HPAweb_C/1296686942137 (Last accessed 12th September 2011)."
- Hunter, P.A., S. Dawson, G.L. French, H. Goossens, P.M. Hawkey, E.J. Kuijper, D. Nathwani, D.J. Taylor, C.J. Teale, R.E. Warren, M.H. Wilcox, N. Woodford, M.W. Wulf and L.J. Piddock, (2010) Antimicrobial-resistant pathogens in animals and man: prescribing, practices and policies. *Journal of Antimicrobial Chemotherapy*. **65** Suppl 1, i3-17.
- Hunter, P.R., D.C. Wilkinson, L.A. Catling and G.C. Barker, (2008) Meta-Analysis of Experimental Data Concerning Antimicrobial Resistance Gene Transfer Rates during Conjugation. *Applied Environmental Microbiology*. **74** (19) 6085-6090.
- Hurley, B.P. and B.A. McCormick, (2003) Translating tissue culture results into animal models: the case of *Salmonella* Typhimurium. *Trends in Microbiology*. **11** (12) 562-569.
- Iredell, J., J. Ellem, Z. Zong and S. Partridge, (2011) The ecology of major resistance plasmids in *E. coli*. 21st European Congress of Clinical Microbiology and Infectious Diseases. Poster P560.
- Isabella, V. and V. Clark, (2011). Deep sequencing-based analysis of the anaerobic stimulon in *Neisseria gonorrhoeae*. *BMC Genomics*. **12** (1) 51.

- Ishii, Y., A. Ohno, H. Taguchi, S. Imajo, M. Ishiguro and H. Matsuzawa, (1995). Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A beta-lactamase isolated from *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. **39** (10) 2269-2275.
- Jacobson, A., (1972) Role of F Pili in the Penetration of Bacteriophage fl. *Journal of Virology*. **10** (4) 835-843.
- Jalasvuori, M., V.-P. Friman, A. Nieminen, J.K.H. Bamford and A. Buckling, (2011) Bacteriophage selection against a plasmid-encoded sex apparatus leads to the loss of antibiotic-resistance plasmids. *Biology Letters*. Jun 1 Epub (as of Sept 2011)
- Jarlier, V., M.-h. Nicolas, G. Fournier and A. Philippon, (1988) Extended Broad-Spectrum β -Lactamases Conferring Transferable Resistance to Newer β -Lactam Agents in *Enterobacteriaceae*: Hospital Prevalence and Susceptibility Patterns. *Reviews of Infectious Diseases*. **10** (4) 867-878.
- Jarrett, P. and M. Stephenson, (1990). Plasmid transfer between strains of *Bacillus thuringiensis* infecting *Galleria mellonella* and *Spodoptera littoralis*. *Applied Environmental Microbiology*. **56** (6) 1608-1614.
- Jevons, M., (1961) "Celbenin"-resistant staphylococci. *BMJ*. **1**, 124-125.
- Jiang, Y., D. Yu, Z. Wei, P. Shen, Z. Zhou and Y. Yu, (2010) Complete Nucleotide Sequence of *Klebsiella pneumoniae* Multidrug Resistance Plasmid pKP048, Carrying *bla*_{KPC-2}, *bla*_{DHA-1}, *qnrB4*, and *armA*. *Antimicrobial Agents and Chemotherapy*. **54** (9) 3967-3969.
- Johnson, J.R., C. Clabots and M.A. Kuskowski, (2008). Multiple-Host Sharing, Long-Term Persistence, and Virulence of *Escherichia coli* Clones from Human and Animal Household Members. *Journal of Clinical Microbiology*. **46** (12) 4078-4082.
- Johnson, R.C. and M.I. Simon, (1985) Hin-mediated site-specific recombination requires two 26 bp recombination sites and a 60 bp recombinational enhancer. *Cell*. **41** (3) 781-791.
- Johnson, T.J., Y.M. Wannemuehler, S.J. Johnson, C.M. Logue, D.G. White, C. Doetkott and L.K. Nolan, (2007). Plasmid replicon typing of commensal and pathogenic *Escherichia coli* isolates. *Applied Environmental Microbiology*. **73** (6) 1976-1983.
- Jones, D. and P.H. Sneath, (1970) Genetic transfer and bacterial taxonomy. *Microbiology and Molecular Biology Reviews*. **34** (1) 40-81.
- Joris, B., J. Ghuysen, G. Dive, A. Renard, O. Dideberg, P. Charlier, J. Frère, J. Kelly, J. Boyington and P. Moews, (1988). The active-site-serine penicillin-recognizing enzymes as members of the Streptomyces R61 DD-peptidase family. *The Biochemical journal*. **250** (2) 313-324.
- Kamachi, K., M. Sota, Y. Tamai, N. Nagata, T. Konda, T. Inoue, E.M. Top and Y. Arakawa, (2006) Plasmid pBP136 from *Bordetella pertussis* represents an ancestral form of IncP-1 β plasmids without accessory mobile elements. *Microbiology*. **152** (12) 3477-3484.
- Kang, H.Y., J. Kim, S.Y. Seol, Y.C. Lee, J.C. Lee and D.T. Cho, (2009) Characterization of conjugative plasmids carrying antibiotic resistance genes encoding 16S rRNA methylase, extended-spectrum beta-lactamase, and/or plasmid-mediated AmpC beta-lactamase. *Journal of Microbiology*. **47** (1) 68-75.

- Kaper, J.B., J.P. Nataro and H.L. Mobley, (2004) Pathogenic *Escherichia coli*. *Nature Reviews Microbiology*. **2** (2) 123-140.
- Karim, A., L. Poirel, S. Nagarajan and P. Nordmann, (2001) Plasmid-mediated extended-spectrum beta-lactamase (CTX-M-3 like) from India and gene association with insertion sequence *ISEcp1*. *FEMS Microbiology Letters*. **201** (2) 237-241.
- Katashkina, J.I., Y. Hara, L.I. Golubeva, I.G. Andreeva, T.M. Kuvaeva and S.V. Mashko, (2009) Use of the lambda Red-recombineering method for genetic engineering of *Pantoea ananatis*. *BMC Molecular Biology*. **10**, 34.
- Kato, A. and K. Mizobuchi, (1994) Evolution of the replication regions of IncI alpha and IncFII plasmids by exchanging their replication control systems. *DNA Research*. **1** (5) 201-212.
- Kim, J., I.K. Bae, S.H. Jeong, C.L. Chang, C.H. Lee and K. Lee, (2011). Characterization of IncF plasmids carrying the *bla*_{CTX-M-14} gene in clinical isolates of *Escherichia coli* from Korea. *Journal of Antimicrobial Chemotherapy*. **66** (6) 1263-8.
- Kim, S.R., N. Funayama and T., (1993) Nucleotide sequence and characterization of the *traABCD* region of IncI1 plasmid R64. *Journal of Bacteriology*. **175** (16) 5035-5042.
- Kim, S.R. and T. Komano, (1992) Nucleotide sequence of the R721 shufflon. *Journal of Bacteriology*. **174** (21) 7053-7058.
- Kiratisin, P. and A. Henprasert, (2010) Resistance phenotype-genotype correlation and molecular epidemiology of *Citrobacter*, *Enterobacter*, *Proteus*, *Providencia*, *Salmonella* and *Serratia* that carry extended-spectrum beta-lactamases with or without plasmid-mediated AmpC beta-lactamase genes in Thailand. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. **105** (1) 46-51.
- Knodler, L.A., A. Bestor, C. Ma, I. Hansen-Wester, M. Hensel, B.A. Vallance and O. Steele-Mortimer, (2005) Cloning Vectors and Fluorescent Proteins Can Significantly Inhibit *Salmonella enterica* Virulence in Both Epithelial Cells and Macrophages: Implications for Bacterial Pathogenesis Studies. *Infection and Immunity*. **73** (10) 7027-7031.
- Kollef, M.H., (2006) Is Antibiotic Cycling the Answer to Preventing the Emergence of Bacterial Resistance in the Intensive Care Unit? *Clinical Infectious Diseases*. **43** (Supplement 2) S82-S88.
- Komano, T., (1999) Shufflons: multiple inversion systems and integrons. *Annual Review of Genetics*. **33**, 171-191.
- Komano, T., S.-R. Kim, T. Yoshida and T. Nisioka, (1994) DNA Rearrangement of the Shufflon Determines Recipient Specificity in Liquid Mating of IncI1 Plasmid R64. *Journal of Molecular Biology*. **243** (1) 6-9.
- Komano, T., S.R. Kim and T. Yoshida, (1995) Mating variation by DNA inversions of shufflon in plasmid R64. *Advances in Biophysics*. **31**, 181-193.
- Komano, T., T. Yoshida, K. Narahara and N. Furuya, (2000) The transfer region of IncI1 plasmid R64: similarities between R64 *tra* and *legionella icm/dot* genes. *Molecular Microbiology*. **35** (6) 1348-1359.

- Labrousse, A., S. Chauvet, C. Couillault, C.L. Kurz and J.J. Ewbank, (2000) *Caenorhabditis elegans* is a model host for *Salmonella* Typhimurium. *Current Biology*. **10** (23) 1543-1545.
- Lan, R. and Reeves, P.R. (2002) *Escherichia coli* in disguise: molecular origins of *Shigella*. *Microbes and Infection*, **4**, 1125-1132
- Langridge, G.C., M.D. Phan, D.J. Turner, T.T. Perkins, L. Parts, J. Haase, I. Charles, D.J. Maskell, S.E. Peters, G. Dougan, J. Wain, J. Parkhill and A.K. Turner, (2009) Simultaneous assay of every *Salmonella* Typhi gene using one million transposon mutants. *Genome Research*. **19** (12) 2308-2316.
- Lartigue, M.F., L. Poirel, D. Aubert and P. Nordmann, (2006) *In vitro* analysis of ISEcp1B-mediated mobilization of naturally occurring beta-lactamase gene *bla*_{CTX-M} of *Kluyvera ascorbata*. *Antimicrobial Agents and Chemotherapy*. **50** (4) 1282-1286.
- Lartigue, M.F., L. Poirel and P. Nordmann, (2004) Diversity of genetic environment of *bla*_{CTX-M} genes. *FEMS Microbiology Letters*. **234** (2) 201-207.
- Lau, S.H., M.E. Kaufmann, D.M. Livermore, N. Woodford, G.A. Willshaw, T. Cheasty, K. Stamper, S. Reddy, J. Cheesbrough, F.J. Bolton, A.J. Fox and M. Upton, (2008) UK epidemic *Escherichia coli* strains A-E, with CTX-M-15 beta-lactamase, all belong to the international O25:H4-ST131 clone. *Journal of Antimicrobial Chemotherapy*. **62** (6) 1241-1244.
- Laupland, K.B., D.L. Church, J. Vidakovich, M. Mucenski and J.D.D. Pitout, (2008) Community-onset extended-spectrum beta-lactamase (ESBL) producing *Escherichia coli*: Importance of international travel. *Journal of Infection*. **57** (6) 441-448.
- Lavigne, J.P., A.B. Blanc-Potard, G. Bourg, D. O'Callaghan and A. Sotto, (2006) *Caenorhabditis elegans*: modèle d'étude *in vivo* de la virulence bactérienne. *Pathologie Biologie*. **54** (8-9) 439-446.
- Lawrence, J.G., H. Ochman and D.L. Hartl, (1992). The Evolution of Insertion Sequences within Enteric Bacteria. *Genetics*. **131** (1) 9-20.
- Lee, C., J. Kim, S.G. Shin and S. Hwang, (2006). Absolute and relative QPCR quantification of plasmid copy number in *Escherichia coli*. *Journal of Biotechnology*. **123** (3) 273-280.
- Lee, C.L., D.S. Ow and S.K. Oh, (2006). Quantitative real-time polymerase chain reaction for determination of plasmid copy number in bacteria. *Journal of Microbiological Methods*. **65** (2) 258-267.
- Lee, S.G., S.H. Jeong, H. Lee, C.K. Kim, Y. Lee, E. Koh, Y. Chong and K. Lee, (2009) Spread of CTX-M-type extended-spectrum beta-lactamases among bloodstream isolates of *Escherichia coli* and *Klebsiella pneumoniae* from a Korean hospital. *Diagnostic Microbiology and Infectious Disease*. **63** (1) 76-80.
- Lee, S.W. and G. Edlin, (1985) Expression of tetracycline resistance in pBR322 derivatives reduces the reproductive fitness of plasmid-containing *Escherichia coli*. *Gene*. **39** (2-3) 173-180.
- Lenski, R., (1998) Bacterial evolution and the cost of antibiotic resistance. *International Microbiology*. **1**, 265 - 270.

- Lenski, R.E., (1998) Bacterial evolution and the cost of antibiotic resistance. *International Microbiology*. **1** (4) 265-270.
- Lenski, R.E., S.C. Simpson and T.T. Nguyen, (1994) Genetic analysis of a plasmid-encoded, host genotype-specific enhancement of bacterial fitness. *Journal of Bacteriology*. **176** (11) 3140-3147.
- Leyton, D.L., J. Sloan, R.E. Hill, S. Doughty and E.L. Hartland, 2003. Transfer region of pO113 from enterohemorrhagic *Escherichia coli*: similarity with R64 and identification of a novel plasmid-encoded autotransporter, EpeA. *Infection and Immunity*. **71** (11) 6307-6319.
- Li, X.Z., M. Mehrotra, S. Ghimire and L. Adewoye, (2007) beta-lactam resistance and beta-lactamases in bacteria of animal origin. *Veterinary Microbiology*. **121** (3-4) 197-214.
- Liebana, E., M. Batchelor, K.L. Hopkins, F.A. Clifton-Hadley, C.J. Teale, A. Foster, L. Barker, E.J. Threlfall and R.H. Davies, (2006) Longitudinal farm study of extended-spectrum beta-lactamase-mediated resistance. *Journal of Clinical Microbiology*. **44** (5) 1630-1634.
- Lim, Y.M., A.J.C. de Groof, M.K. Bhattacharjee, D.H. Figurski and E.A. Schon, (2008) Bacterial Conjugation in the Cytoplasm of Mouse Cells. *Infection and Immunity*. **76** (11) 5110-5119.
- Lin, A., J. Jimenez, J. Derr, P. Vera, M.L. Manapat, K.M. Esvelt, L. Villanueva, D.R. Liu and I.A. Chen, (2011) Inhibition of Bacterial Conjugation by Phage M13 and Its Protein g3p: Quantitative Analysis and Model. *PLoS ONE*. **6** (5) e19991.
- Livermore, D.M., (2008) Defining an extended-spectrum beta-lactamase. *Clinical Microbiology and Infection*. **14**, Suppl 1, 3-10.
- Llarrull, L.I., S.A. Testero, J.F. Fisher and S. Mobashery, (2011) The future of the beta-lactams. *Current Opinion in Microbiology*. **13** (5) 551-557.
- Lo, W.U., P.L. Ho, K.H. Chow, E.L. Lai, F. Yeung and S.S. Chiu, (2010) Fecal carriage of CTXM type extended-spectrum beta-lactamase-producing organisms by children and their household contacts. *Journal of infection*. **60** (4) 286-92.
- Loftie-Eaton, W. and D.E. Rawlings, (2010) Evolutionary Competitiveness of Two Natural Variants of the IncQ-Like Plasmids, pRAS3.1 and pRAS3.2. *Journal of Bacteriology*. **192** (23) 6182-6190.
- Lujan, S.A., L.M. Guogas, H. Ragonese, S.W. Matson and M.R. Redinbo, (2007) Disrupting antibiotic resistance propagation by inhibiting the conjugative DNA relaxase. *Proceedings of the National Academy of Sciences USA*. **104** (30) 12282-12287.
- Luo, N., S. Pereira, O. Sahin, J. Lin, S. Huang, L. Michel and Q. Zhang, (2005) Enhanced *in vivo* fitness of fluoroquinolone-resistant *Campylobacter jejuni* in the absence of antibiotic selection pressure. *Proceedings of the National Academy of Sciences USA*. **102** (3) 541-546.
- Ma, L., Y. Ishii, F.Y. Chang, K. Yamaguchi, M. Ho and L.K. Siu, (2002) CTX-M-14, a plasmid-mediated CTX-M type extended-spectrum beta-lactamase isolated from *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. **46** (6) 1985-1988.

- Macvanin, M., A. Ballagi and D. Hughes, (2004). Fusidic Acid-Resistant Mutants of *Salmonella enterica* Serovar Typhimurium Have Low Levels of Heme and a Reduced Rate of Respiration and Are Sensitive to Oxidative Stress. *Antimicrobial Agents and Chemotherapy*. **48** (10) 3877-3883.
- Maltezou, H.C., (2009). Metallo-beta-lactamases in Gram-negative bacteria: introducing the era of pan-resistance? *International Journal of Antimicrobial Agents*. **33** (5) 405.e401-405.e407.
- Mankovich, J.A., C.H. Hsu and J. Konisky, (1986). DNA and amino acid sequence analysis of structural and immunity genes of colicins Ia and Ib. *Journal of Bacteriology*. **168** (1) 228-236.
- Marcade, G., C. Deschamps, A. Boyd, V. Gautier, B. Picard, C. Branger, E. Denamur and G. Arlet, (2008). Replicon typing of plasmids in *Escherichia coli* producing extended-spectrum beta-lactamases. *Journal of Antimicrobial Chemotherapy*. **63** (1) 67-71.
- March, A., R. Aschbacher, H. Dhanji, D.M. Livermore, A. Böttcher, F. Sleghe, S. Maggi, M. Noale, C. Larcher and N. Woodford, (2010) Colonization of residents and staff of a long-term-care facility and adjacent acute-care hospital geriatric unit by multiresistant bacteria. *Clinical Microbiology and Infection*. **16** (7) 934-944.
- Marciano, D.C., O.Y. Karkouti and T. Palzkill, (2007) A fitness cost associated with the antibiotic resistance enzyme SME-1 beta-lactamase. *Genetics*. **176** (4) 2381-2392.
- Marianelli, C., F. Ciuchini, M. Tarantino, P. Pasquali and R. Adone, (2004) Genetic bases of the rifampin resistance phenotype in *Brucella spp.* *Journal of Clinical Microbiology*. **42** (12) 5439-5443.
- Massova, I. and S. Mobashery, (1998) Kinship and diversification of bacterial penicillin-binding proteins and beta-lactamases. *Antimicrobial Agents and Chemotherapy*. **42** (1) 1-17.
- Matsumoto, Y., F. Ikeda, T. Kamimura, Y. Yokota and Y. Mine, (1988) Novel plasmid-mediated beta-lactamase from *Escherichia coli* that inactivates oxyimino-cephalosporins. *Antimicrobial Agents and Chemotherapy*. **32** (8) 1243-1246.
- May, T., K. Tsuruta and S. Okabe, (2010) Exposure of conjugative plasmid carrying *Escherichia coli* biofilms to male-specific bacteriophages. *ISME Journal*. **5** (4) 771-775.
- McDermott, P.J., P. Gowland and P.C. Gowland, (1993). Adaptation of *Escherichia coli* growth rates to the presence of pBR322. *Letters in Applied Microbiology*. **17** (3) 139-143.
- Merryweather, A., C. Rees, N.M. Smith and B.M. Wilkins, (1986) Role of *sog* polypeptides specified by plasmid Collb-P9 and their transfer between conjugating bacteria. *EMBO Journal*. **5** (11) 3007-3012.
- Meynell, E. and N. Datta, (1966) The relation of resistance transfer factors to the F-factor (sex-factor) of *Escherichia coli* K12. *Genetical Research*. **7** (1) 134-140.
- Miles, A.A., S.S. Misra and J.O. Irwin, (1938) The estimation of the bactericidal power of the blood. *The Journal of Hygiene*. **38** (6) 732-749.

- Miller, C.A. and S.N. Cohen, (1993) The partition (*par*) locus of pSC101 is an enhancer of plasmid incompatibility. *Molecular Microbiology*. **9** (4) 695-702.
- Miro, E., B. Mirelis, F. Navarro, A. Rivera, R.J. Mesa, M.C. Roig, L. Gomez and P. Coll, (2005) Surveillance of extended-spectrum beta-lactamases from clinical samples and faecal carriers in Barcelona, Spain. *Journal of Antimicrobial Chemotherapy*. **56** (6) 1152-1155.
- Mlynarczyk, A., K. Szymanek, A. Sawicka-Grzelak, J. Pazik, T. Buczkowska, M. Durlík, B. Lagiewska, M. Pacholczyk, A. Chmura, L. Paczek and G. Mlynarczyk, (2009) CTX-M and TEM as Predominant Types of Extended Spectrum beta-lactamases Among *Serratia marcescens* Isolated From Solid Organ Recipients. *Transplantation Proceedings*. **41** (8) 3253-3255.
- Modi, R.I. and J. Adams, (1991). Coevolution in bacterial-plasmid populations. *Evolution*. **43** (3) 656 - 667.
- Molin, S. and T. Tolker-Nielsen, (2003) Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure. *Current Opinion in Biotechnology*. **14** (3) 255-261.
- Montgomery, K., L. Raymundo, Jr. and W.L. Drew, (1979) Chromogenic cephalosporin spot test to detect beta-lactamase in clinically significant bacteria. *Journal of Clinical Microbiology*. **9** (2) 205-207.
- Mora, A., M. Blanco, C. López, R. Mamani, J.E. Blanco, M.P. Alonso, F. García-Garrote, G. Dahbi, A. Herrera, A. Fernández, B. Fernández, A. Agulla, G. Bou and J. Blanco, (2010) Emergence of clonal groups O1:HNM-D-ST59, O15:H1-D-ST393, O20:H34/HNM-D-ST354, O25b:H4-B2-ST131 and ONT:H21,42-B1-ST101 among CTX-M-14-producing *Escherichia coli* clinical isolates in Galicia, northwest Spain. *International Journal of Antimicrobial Agents*. **37** (1) 16-21.
- Morck, D.W., T.J. Raybould, S.D. Acres, L.A. Babiuk, J. Nelligan and J.W. Costerton, (1987) Electron microscopic description of glycocalyx and fimbriae on the surface of *Pasteurella haemolytica*-A1. *Canadian Journal of Veterinary Research*. **51** (1) 83-88.
- Moreno, A., H. Bello, D. Guggiana, M. Dominguez and G. Gonzalez, (2008) Extended-spectrum beta-lactamases belonging to CTX-M group produced by *Escherichia coli* strains isolated from companion animals treated with enrofloxacin. *Veterinary Microbiology*. **129** (1-2) 203-208.
- Morosini, M.I., J.A. Ayala, F. Baquero, J.L. Martinez and J. Blazquez, (2000) Biological Cost of AmpC Production for *Salmonella enterica* Serotype Typhimurium. *Antimicrobial Agents and Chemotherapy*. **44** (11) 3137-3143.
- Morris, C., C.M.C. Yip, I.S.M. Tsui, D.K.H. Wong and J. Hackett, (2003). The Shufflon of *Salmonella enterica* Serovar Typhi Regulates Type IVB Pilus-Mediated Bacterial Self-Association. *Infection and Immunity*. **71** (3) 1141-1146.

- Moya, B., C. Juan, S. Alberti, J.L. Perez and A. Oliver, (2008). Benefit of Having Multiple *ampD* Genes for Acquiring beta-Lactam Resistance without Losing Fitness and Virulence in *Pseudomonas aeruginosa*. *Antimicrobial Agents and Chemotherapy*. **52** (10) 3694-3700.
- Mroczkowska, J.E. and M. Barlow, (2008) Fitness Trade-Offs in *bla*_{TEM} Evolution. *Antimicrobial Agents and Chemotherapy*. **52** (7) 2340-2345.
- Mulvey, M.R., E. Susky, M. McCracken, D.W. Morck and R.R. Read, (2009). Similar cefoxitin-resistance plasmids circulating in *Escherichia coli* from human and animal sources. *Veterinary Microbiology*. **134** (3-4) 279-287.
- Munday, C.J., G.M. Whitehead, N.J. Todd, M. Campbell and P.M. Hawkey, (2004). Predominance and genetic diversity of community- and hospital-acquired CTX-M extended-spectrum beta-lactamases in York, UK. *Journal of Antimicrobial Chemotherapy*. **54** (3) 628-633.
- Navarro, F., R.J. Mesa, E. Miro, L. Gomez, B. Mirelis and P. Coll, (2007). Evidence for convergent evolution of CTX-M-14 ESBL in *Escherichia coli* and its prevalence. *FEMS Microbiology Letters*. **273** (1) 120-123.
- Navarro, F. and E. Miro, (2002) Update on CTX-M-type beta lactamases. *Reviews in Medical Microbiology*. **13** (2) 63-73.
- NHS, (2011) "Data from the National Prescribing centre. Common infections: Data focus. Commentary available at <http://www.npci.org.uk/therapeutics> (Last accessed September 2011)".
- Nielsen, A.K. and K. Gerdes, (1995) Mechanism of post-segregational killing by *hok*-homologue *pnd* of plasmid R483: Two translational control elements in the *pnd* mRNA. *Journal of Molecular Biology*. **249** (2) 270-282.
- Nielsen, A.K., P. Thorsted, T. Thisted, E.G.H. Wagner and K. Gerdes, (1991) The rifampicin-inducible genes *srn6* from F and *pnd* from R483 are regulated by antisense RNAs and mediate plasmid maintenance by killing of plasmid-free segregants. *Molecular Microbiology*. **5** (8) 1961-1973.
- Nishino, K., T. Latifi and E.A. Groisman, (2006) Virulence and drug resistance roles of multidrug efflux systems of *Salmonella enterica* serovar Typhimurium. *Molecular Microbiology*. **59** (1) 126-141.
- Norberg, P., M. Bergstrom, V. Jethava, D. Dubhashi and M. Hermansson, (2011) The IncP1 plasmid backbone adapts to different host bacterial species and evolves through homologous recombination. *Nature Communication*. **2**, 268.
- Nordmann, P., M.F. Lartigue and L. Poirel, (2008) Beta-lactam induction of ISEcp1B-mediated mobilization of the naturally occurring *bla*_{CTX-M} beta-lactamase gene of *Kluyvera ascorbata*. *FEMS Microbiology Letters*. **288** (2) 247-249.
- Normark, B.H. and S. Normark, (2002). Evolution and spread of antibiotic resistance. *Journal of Internal Medicine*. **252** (2) 91-106.

- Novais, A., R. Canton, R. Moreira, L. Peixe, F. Baquero and T.M. Coque, (2007). Emergence and dissemination of *Enterobacteriaceae* isolates producing CTX-M-1-like enzymes in Spain are associated with IncFII (CTX-M-15) and broad-host-range (CTX-M-1, -3, and -32) plasmids. *Antimicrobial Agents and Chemotherapy*. **51** (2) 796-799.
- Novais, A., I. Comas, F. Baquero, R. Cantón, T. Coque, A. Moya, F. González-Candelas and J. Galán, (2010) Evolutionary Trajectories of Beta-Lactamase CTX-M-1 Cluster Enzymes: Predicting Antibiotic Resistance. *PLoS Pathogens*. **6** (1) e1000735.
- Novick, R.P., (1987) Plasmid incompatibility. *Microbiology Reviews*. **51** (4) 381-395.
- Novick, R.P., R.C. Clowes, S.N. Cohen, R. Curtiss, 3rd, N. Datta and S. Falkow, (1976) Uniform nomenclature for bacterial plasmids: a proposal. *Bacteriological Reviews*. **40** (1) 168-189.
- Novotny, C., W.S. Knight and C.C. Brinton, Jr., (1968) Inhibition of Bacterial Conjugation by Ribonucleic Acid and Deoxyribonucleic Acid Male-Specific Bacteriophages. *Journal of Bacteriology*. **95** (2) 314-326.
- O'Brien, S.J., G.K. Adak and C. Gilham, (2001) Contact with farming environment as a major risk factor for Shiga toxin (Vero cytotoxin)-producing *Escherichia coli* O157 infection in humans. *Emerging Infectious Diseases*. **7** (6) 1049-1051.
- O'Brien, T.F., (2002) Emergence, Spread, and Environmental Effect of Antimicrobial Resistance: How Use of an Antimicrobial Anywhere Can Increase Resistance to Any Antimicrobial Anywhere else. *Clinical Infectious Diseases*. **34** (Supplement 3) S78-S84.
- Ogbolu, D.O., O.A. Daini, A. Ogunledun, A.O. Alli and M.A. Webber, (2011) High levels of multidrug resistance in clinical isolates of Gram-negative pathogens from Nigeria. *International Journal of Antimicrobial Agents*. **37** (1) 62-66.
- Olson, A.B., M. Silverman, D.A. Boyd, A. McGeer, B.M. Willey, V. Pong-Porter, N. Daneman and M.R. Mulvey, (2005) Identification of a progenitor of the CTX-M-9 group of extended-spectrum beta-lactamases from *Kluyvera georgiana* isolated in Guyana. *Antimicrobial Agents and Chemotherapy*. **49** (5) 2112-2115.
- Osborn, A.M., F.M. da Silva Tatley, L.M. Steyn, R.W. Pickup and J.R. Saunders, (2000) Mosaic plasmids and mosaic replicons: evolutionary lessons from the analysis of genetic diversity in IncFII-related replicons. *Microbiology*. **146** (Pt 9) 2267-2275.
- Oteo, J., M. Perez-Vazquez and J. Campos, (2010) Extended-spectrum beta-lactamase producing *Escherichia coli*: changing epidemiology and clinical impact. *Current Opinion in Infectious Diseases*. **23** (4) 320-326.
- Pai, H., E.H. Choi, H.J. Lee, J.Y. Hong and G.A. Jacoby, (2001) Identification of CTX-M-14 extended-spectrum beta-lactamase in clinical isolates of *Shigella sonnei*, *Escherichia coli*, and *Klebsiella pneumoniae* in Korea. *Journal of Clinical Microbiology*. **39** (10) 3747-3749.
- Pallecchi, L., A. Bartoloni, C. Fiorelli, A. Mantella, T. Di Maggio, H. Gamboa, E. Gotuzzo, G. Kronvall, F. Paradisi and G.M. Rossolini, (2007) Rapid dissemination and diversity of CTX-M extended-spectrum beta-lactamase genes in commensal *Escherichia coli* isolates from healthy children from low-resource settings in Latin America. *Antimicrobial Agents and Chemotherapy*. **51** (8) 2720-2725.

- Palmer, C., E.M. Bik, D.B. DiGiulio, D.A. Relman and P.O. Brown, (2007) Development of the Human Infant Intestinal Microbiota. *PLoS Biology*. **5** (7) e177.
- Papagiannitsis, C.C., L.S. Tzouveleakis, S.D. Kotsakis, E. Tzelepi and V. Miriagou, (2011) Sequence of pR3521, an IncB Plasmid from *Escherichia coli* Encoding ACC-4, SCO-1, and TEM-1 beta-Lactamases. *Antimicrobial Agents and Chemotherapy*. **55** (1) 376-381.
- Parsek, M.R. and P.K. Singh, (2003). Bacterial biofilms: an emerging link to disease pathogenesis. *Annual Review of Microbiology*. **57** 677-701.
- Paterson, D.L. and R.A. Bonomo, (2005). Extended-spectrum beta-lactamases: a clinical update. *Clinical Microbiology Reviews*. **18** (4) 657-686.
- Paulander, W., S. Maisnier-Patin and D.I. Andersson, (2009) The Fitness Cost of Streptomycin Resistance Depends on *rpsL* Mutation, Carbon Source and RpoS. *Genetics*. **183** (2) 539-546.
- Pavan, M.E., R.J. Franco, J.M. Rodriguez, P. Gadaleta, S.L. Abbott, J.M. Janda and J. Zorzopulos, (2005) Phylogenetic relationships of the genus *Kluyvera*: transfer of *Enterobacter intermedius* Izard *et al.*, 1980 to the genus *Kluyvera* as *Kluyvera intermedia* comb. nov. and reclassification of *Kluyvera cochleae* as a later synonym of *K. intermedia*. *International Journal of Systematic and Evolutionary Microbiology*. **55** (Pt 1) 437-442.
- Peirano, G. and J.D.D. Pitout, (2010) Molecular epidemiology of *Escherichia coli* producing CTX-M beta-lactamases: the worldwide emergence of clone ST131 O25:H4. *International Journal of Antimicrobial Agents*. **35** (4) 316-321.
- Pennington, H., (2010) *Escherichia coli* O157. *The Lancet*. **376** (9750) 1428-1435.
- Perez, F., A. Endimiani, K.M. Hujer and R.A. Bonomo, (2007). The continuing challenge of ESBLs. *Current Opinion in Pharmacology*. **7** (5) 459-469.
- Petersen, A., F.M. Aarestrup and J.E. Olsen, (2009). The *in vitro* fitness cost of antimicrobial resistance in *Escherichia coli* varies with the growth conditions. *FEMS Microbiology Letters*. **299** (1) 53-59.
- Petrocheilou, V., J. Grinsted and M.H. Richmond, (1976) R-Plasmid Transfer *In vivo* in the Absence of Antibiotic Selection Pressure. *Antimicrobial Agents and Chemotherapy*. **10** (4) 753-761.
- Petroni, A., A. Corso, R. Melano, M.L. Cacace, A.M. Bru, A. Rossi and M. Galas, (2002) Plasmidic extended-spectrum beta-lactamases in *Vibrio cholerae* O1 El Tor isolates in Argentina. *Antimicrobial Agents and Chemotherapy*. **46** (5) 1462-1468.
- Pfeifer, Y., A. Cullik and W. Witte, (2010). Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *International Journal of Medical Microbiology*. **300** (6) 371-379.
- Phan, M.-D., C. Kidgell, S. Nair, K.E. Holt, A.K. Turner, J. Hinds, P. Butcher, F.J. Cooke, N.R. Thomson, R. Titball, Z.A. Bhutta, R. Hasan, G. Dougan and J. Wain, (2009) Variation in *Salmonella enterica* Seroovar Typhi IncHI1 Plasmids during the Global Spread of Resistant Typhoid Fever. *Antimicrobial Agents and Chemotherapy*. **53** (2) 716-727.

- Phan, M.D. (2009) Analysis of IncHI1 plasmids in *Salmonella enterica* serovar Typhi. Darwin College. University of Cambridge. Ph.D. thesis (unpublished).
- Piddock, L.J., R.N. Walters, Y.F. Jin, H.L. Turner, D.M. Gascoyne-Binzi and P.M. Hawkey, (1997) Prevalence and mechanism of resistance to 'third-generation' cephalosporins in clinically relevant isolates of *Enterobacteriaceae* from 43 hospitals in the UK, 1990-1991. *Journal of Antimicrobial Chemotherapy*. **39** (2) 177-187.
- Pinto, A.C., H.P. Melo-Barbosa, A. Miyoshi, A. Silva and V. Azevedo, (2011) Application of RNA-seq to reveal the transcript profile in bacteria. *Genetic and Molecular Research*. **10** (3) 1707-1718.
- Pitout, J.D., (2008). Multiresistant *Enterobacteriaceae*: new threat of an old problem. *Expert Review of Anti-Infective Therapy*. **6** (5) 657-669.
- Pitout, J.D., L. Campbell, D.L. Church, D.B. Gregson and K.B. Laupland, (2009) Molecular Characteristics of Travel-Related Extended-Spectrum beta-lactamase producing *Escherichia coli* from the Calgary Health Region. *Antimicrobial Agents and Chemotherapy*. **53** (6) 2539-43.
- Pitout, J.D., D.B. Gregson, L. Campbell and K.B. Laupland, (2009). Molecular characteristics of extended-spectrum-beta-lactamase-producing *Escherichia coli* isolates causing bacteremia in the Calgary Health Region from 2000 to 2007: emergence of clone ST131 as a cause of community-acquired infections. *Antimicrobial Agents and Chemotherapy*. **53** (7) 2846-2851.
- Pitout, J.D., N.D. Hanson, D.L. Church and K.B. Laupland, (2004). Population-based laboratory surveillance for *Escherichia coli*-producing extended-spectrum beta-lactamases: importance of community isolates with *bla*_{CTX-M} genes. *Clinical Infectious Diseases*. **38** (12) 1736-1741.
- Poirel, L., J.W. Decousser and P. Nordmann, (2003) Insertion sequence *ISEcp1B* is involved in expression and mobilization of a *bla*_{CTX-M} beta-lactamase gene. *Antimicrobial Agents and Chemotherapy*. **47** (9) 2938-2945.
- Poirel, L., P. Kampfer and P. Nordmann, (2002) Chromosome-encoded Ambler class A beta-lactamase of *Kluyvera georgiana*, a probable progenitor of a subgroup of CTX-M extended-spectrum beta-lactamases. *Antimicrobial Agents and Chemotherapy*. **46** (12) 4038-4040.
- Poirel, L., M.F. Lartigue, J.W. Decousser and P. Nordmann, (2005) *ISEcp1B*-mediated transposition of *bla*_{CTX-M} in *Escherichia coli*. *Antimicrobial Agents and Chemotherapy*. **49** (1) 447-450.
- Poirel, L., T. Naas, I. Le Thomas, A. Karim, E. Bingen and P. Nordmann, (2001) CTX-M-type extended-spectrum beta-lactamase that hydrolyzes ceftazidime through a single amino acid substitution in the omega loop. *Antimicrobial Agents and Chemotherapy*. **45** (12) 3355-3361.
- Poole, T.L., J.L. McReynolds, T.S. Edrington, J.A. Byrd, T.R. Callaway and D.J. Nisbet, (2006) Effect of flavophospholipol on conjugation frequency between *Escherichia coli*

- donor and recipient pairs *in vitro* and in the chicken gastrointestinal tract. *Journal of Antimicrobial Chemotherapy*. **58** (2) 359-366.
- Pope, C.F., S.H. Gillespie, J.E. Moore and T.D. McHugh, (2010) Approaches to measure the fitness of *Burkholderia cepacia* complex isolates. *Journal of Medical Microbiology*. **59** (6) 679-686.
- Poppe, C., L.C. Martin, C.L. Gyles, R. Reid-Smith, P. Boerlin, S.A. McEwen, J.F. Prescott and K.R. Forward, (2005) Acquisition of resistance to extended-spectrum cephalosporins by *Salmonella enterica* subsp. *enterica* serovar Newport and *Escherichia coli* in the turkey poult intestinal tract. *Applied Environmental Microbiology*. **71** (3) 1184-1192.
- Porter, C.K., M.S. Riddle, D.R. Tribble, A. Louis Bougeois, R. McKenzie, S.D. Isidean, P. Sebeny and S.J. Savarino, (2011) A systematic review of experimental infections with enterotoxigenic *Escherichia coli* (ETEC). *Vaccine*. **29** (35) 5869-5885.
- Praszkier, J., T. Wei, K. Siemering and J. Pittard, (1991) Comparative analysis of the replication regions of IncB, IncK, and IncZ plasmids. *Journal of Bacteriology*. **173** (7) 2393-2397.
- Prieur, D., G. Erauso, C. Geslin, S. Lucas, M. Gaillard, A. Bidault, A.C. Mattenet, K. Rouault, D. Flament, P. Forterre and M. Le Romancer, (2004) Genetic elements of Thermococcales. *Biochemical Society Transactions*. **32** (Pt 2) 184-187.
- Radice, M., P. Power, J. Di Conza and G. Gutkind, (2002) Early dissemination of CTX-M-derived enzymes in South America. *Antimicrobial Agents and Chemotherapy*. **46** (2) 602-604.
- Rankin, D.J., E.P.C. Rocha and S.P. Brown, (2011) What genes are carried on mobile elements, and why? *Heredity*. **106**, 1 - 10.
- Rasko, D.A., M.J. Rosovitz, G.S.A. Myers, E.F. Mongodin, W.F. Fricke, P. Gajer, J. Crabtree, M. Sebahia, N.R. Thomson, R. Chaudhuri, I.R. Henderson, V. Sperandio and J. Ravel, (2008) The Pangenome Structure of *Escherichia coli*: Comparative Genomic Analysis of *E. coli* Commensal and Pathogenic Isolates. *Journal of Bacteriology*. **190** (20) 6881-6893.
- Rees, C.E. and B.M. Wilkins, (1989) Transfer of *tra* proteins into the recipient cell during bacterial conjugation mediated by plasmid Collb-P9. *Journal of Bacteriology*. **171** (6) 3152-3157.
- Reinthal, F.F., G. Feierl, H. Galler, D. Haas, E. Leitner, F. Mascher, A. Melkes, J. Posch, I. Winter, G. Zarfel and E. Marth, (2009) ESBL-producing *E. coli* in Austrian sewage sludge. *Water Research*. **44** (6) 1981-5.
- Richmond, M.H. and R.B. Sykes, (1973) The beta-lactamases of gram-negative bacteria and their possible physiological role. *Advances in Microbial Physiology*. **9**, 31-88.
- Rodriguez-Bano, J. and A. Pascual, (2008) Clinical significance of extended-spectrum beta-lactamases. *Expert Review of Anti-Infective Therapy*. **6** (5) 671-683.
- Rodriguez-Bano, J., E. Picon, P. Gijon, J.R. Hernandez, M. Ruiz, C. Pena, M. Almela, B. Almirante, F. Grill, J. Colomina, M. Gimenez, A. Oliver, J.P. Horcajada, G. Navarro, A. Coloma and A. Pascual, (2010) Community-Onset Bacteremia Due to Extended-Spectrum

- beta-lactamase-Producing *Escherichia coli*: Risk Factors and Prognosis. *Clinical Infectious Diseases*. **50** (1) 40-48.
- Rodriguez, M.M., P. Power, M. Radice, C. Vay, A. Famiglietti, M. Galleni, J.A. Ayala and G. Gutkind, (2004) Chromosome-encoded CTX-M-3 from *Kluyvera ascorbata*: a possible origin of plasmid-borne CTX-M-1-derived cefotaximases. *Antimicrobial Agents and Chemotherapy*. **48** (12) 4895-4897.
- Rogers, B.A., H.E. Sidjabat and D.L. Paterson, (2011) *Escherichia coli* O25b-ST131: a pandemic, multiresistant, community-associated strain. *Journal of Antimicrobial Chemotherapy*. **66** (1) 1-14.
- Rolinson, G., (1998) Forty years of beta-lactam research. *Journal of Antimicrobial Chemotherapy*. **41**, 589-603.
- Ronald, A., (2002) The etiology of urinary tract infection: traditional and emerging pathogens. *American Journal of Medicine*. **113**, Suppl 1A, 14S-19S.
- Rooney, P.J., M.C. O'Leary, A.C. Loughrey, M. McCalmont, B. Smyth, P. Donaghy, M. Badri, N. Woodford, E. Karisik and D.M. Livermore, (2009). Nursing homes as a reservoir of extended-spectrum beta-lactamase (ESBL)-producing ciprofloxacin-resistant *Escherichia coli*. *Journal of Antimicrobial Chemotherapy*. **64** (3) 635-641.
- Rossolini, G.M., M.M. D'Andrea and C. Mugnaioli, (2008) The spread of CTX-M-type extended-spectrum beta-lactamases. *Clinical Microbiology and Infection*. **14**, Suppl 1, 33-41.
- Rozkov, A., C.A. Avignone-Rossa, P.F. Ertl, P. Jones, R.D. O'Kennedy, J.J. Smith, J.W. Dale and M.E. Bushell, (2004). Characterization of the metabolic burden on *Escherichia coli* DH1 cells imposed by the presence of a plasmid containing a gene therapy sequence. *Biotechnology and Bioengineering*. **88** (7) 909-915.
- Ruppe, E., S. Hem, S. Lath, V. Gautier, F. Arieu, J.L. Sarthou, D. Monchy and G. Arlet, (2009) CTX-M beta-lactamases in *Escherichia coli* from community-acquired urinary tract infections, Cambodia. *Emerging Infectious Diseases*. **15** (5) 741-748.
- Sadowski, P.L., B.C. Peterson, D.N. Gerding and P.P. Cleary, (1979) Physical Characterization of Ten R Plasmids Obtained from an Outbreak of Nosocomial *Klebsiella pneumoniae* Infections. *Antimicrobial Agents and Chemotherapy*. **15** (4) 616-624.
- Saitou, N. and M. Nei, (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution*. **4** (4) 406-425.
- Sampei, G.-i., N. Furuya, K. Tachibana, Y. Saitou, T. Suzuki, K. Mizobuchi and T. Komano, (2010) Complete genome sequence of the incompatibility group I1 plasmid R64. *Plasmid*. **64** (2) 92-103.
- Sanchez, C., (2011) Cellular microbiology: bacterial networking. *Nature Reviews Microbiology*. **9** (4) 229.
- Sanderson, K.E., (1976) Genetic Relatedness in the Family *Enterobacteriaceae*. *Annual Review of Microbiology*. **30** (1) 327-349.

- Sarria, J.C., A.M. Vidal and R.C. Kimbrough, (2001) Infections caused by *Kluyvera* species in humans. *Clinical Infectious Diseases*. **33** (7) E69-74.
- Sasaki, T., I. Hirai, M. Niki, T. Nakamura, C. Komalamisra, W. Maipanich, T. Kusolsuk, S. Sa-nguankiat, S. Pubampen and Y. Yamamoto, (2010) High prevalence of CTX-M beta-lactamase-producing *Enterobacteriaceae* in stool specimens obtained from healthy individuals in Thailand. *Journal of Antimicrobial Chemotherapy*. **65** (4) 666-668.
- Sauvage, E., F. Kerff, M. Terrak, J.A. Ayala and P. Charlier, (2008) The penicillin-binding proteins: structure and role in peptidoglycan biosynthesis. *FEMS Microbiology Reviews*. **32** (2) 234-258.
- Savkovic, S.D., J. Villanueva, J.R. Turner, K.A. Matkowskyj and G. Hecht, (2005) Mouse model of enteropathogenic *Escherichia coli* infection. *Infection and Immunity*. **73** (2) 1161-1170.
- Schmidt, T., K. Friehs and E. Flaschel, (1996) Rapid determination of plasmid copy number. *Journal of Biotechnology*. **49** (1-3) 219-229.
- Schoevaerdt, D., P. Bogaerts, A. Grimmelprez, M. de Saint-Hubert, B. Delaere, J. Jamart, C. Swine and Y. Glupczynski, (2011) Clinical profiles of patients colonized or infected with extended-spectrum beta-lactamase producing *Enterobacteriaceae* isolates: a 20 month retrospective study at a Belgian University Hospital. *BMC Infectious Diseases*. **11** (1) 12.
- Shah, N., H.L. DuPont and D.J. Ramsey, (2009) Global Etiology of Travelers Diarrhea: Systematic Review from 1973 to the Present. *The American Journal of Tropical Medicine and Hygiene*. **80** (4) 609-614.
- Shaw, W.V., L.C. Sands and N. Datta, (1972) Hybridization of variants of chloramphenicol acetyltransferase specified by *fi* + and *fi* - R factors. *Proceedings of the National Academy of Sciences of the USA*. **69** (10) 3049-3053.
- Sheldon, T., (2010) Dutch doctors warn that the overuse of antibiotics in farming is increasing resistance. *BMJ*. **341**, 5677.
- Shen, P., Y. Jiang, Z. Zhou, J. Zhang, Y. Yu and L. Li, (2008) Complete nucleotide sequence of pKP96, a 67 850 bp multiresistance plasmid encoding *qnrA1*, *aac(6')*-Ib-cr and *bla*_{CTX-M-24} from *Klebsiella pneumoniae*. *Journal of Antimicrobial Chemotherapy*. **62** (6) 1252-1256.
- Sherley, M., D.M. Gordon and P.J. Collignon, (2003) Species differences in plasmid carriage in the *Enterobacteriaceae*. *Plasmid*. **49** (1) 79-85.
- Shimoda, E., T. Muto, T. Horiuchi, N. Furuya and T. Komano, (2008) Novel class of mutations of *pilS* mutants, encoding plasmid R64 type IV prepilin: interface of PilS-PilV interactions. *Journal of Bacteriology*. **190** (4) 1202-1208.
- Shorr, A.F., S.T. Micek, E.C. Welch, J.A. Doherty, R.M. Reichley and M.H. Kollef, (2011) Inappropriate antibiotic therapy in Gram-negative sepsis increases hospital length of stay. *Critical Care Medicine*. **39** (1) 46-51.
- Silberstein, Z. and A. Cohen, (1987) Synthesis of linear multimers of OriC and pBR322 derivatives in *Escherichia coli* K-12: role of recombination and replication functions. *Journal of Bacteriology*. **169** (7) 3131-3137.

- Silva, R.F., S.C.M. Mendonca, L.M. Carvalho, A.M. Reis, I. Gordo, S. Trindade and F. Dionisio, (2011) Pervasive Sign Epistasis between Conjugative Plasmids and Drug-Resistance Chromosomal Mutations. *PLoS Genetics*. **7** (7) e1002181.
- Skurnik, D., R. Ruimy, A. Andremont, C. Amorin, P. Rouquet, B. Picard and E. Denamur, (2006) Effect of human vicinity on antimicrobial resistance and integrons in animal faecal *Escherichia coli*. *Journal of Antimicrobial Chemotherapy*. **57** (6) 1215-1219.
- Slater, F.R., M.J. Bailey, A.J. Tett and S.L. Turner, (2008) Progress towards understanding the fate of plasmids in bacterial communities. *FEMS Microbiology Ecology*. **66** (1) 3-13.
- Smet, A., A. Martel, D. Persoons, J. Dewulf, M. Heyndrickx, B. Catry, L. Herman, F. Haesebrouck and P. Butaye, (2008) Diversity of extended-spectrum beta-lactamases and class C beta-lactamases among cloacal *Escherichia coli* Isolates in Belgian broiler farms. *Antimicrobial Agents and Chemotherapy*. **52** (4) 1238-1243.
- Smet, A., A. Martel, D. Persoons, J. Dewulf, M. Heyndrickx, A. Cloeckeaert, K. Praud, G. Claeys, B. Catry, L. Herman, F. Haesebrouck and P. Butaye, (2009) Comparative analysis of extended-spectrum beta-lactamase-carrying plasmids from different members of *Enterobacteriaceae* isolated from poultry, pigs and humans: evidence for a shared beta-lactam resistance gene pool? *Journal of Antimicrobial Chemotherapy*. **63** (6) 1286-8.
- Smillie, C., M.P. Garcillan-Barcia, M.V. Francia, E.P.C. Rocha and F. de la Cruz, (2010) Mobility of Plasmids. *Microbiology and Molecular Biology Reviews*. **74** (3) 434-452.
- Smith, C.A. and C.M. Thomas, (1983) Deletion mapping of *kil* and *kor* functions in the *trfA* and *trfB* regions of broad host range plasmid RK2. *Molecular and General Genetics*. **190** (2) 245-254.
- Smith, H.W., (1975) Persistence of tetracycline resistance in pig *E. coli*. *Nature*. **258** (5536) 628-630.
- Smith, M.A. and M.J. Bidochka, (1998) Bacterial fitness and plasmid loss: the importance of culture conditions and plasmid size. *Canadian Journal of Microbiology*. **44** (4) 351-355.
- Soleimanian, S., N.C. Gordon and D.W. Wareham, (2011) Polymicrobial necrotizing fasciitis involving enterobacteria producing CTX-M-15 extended-spectrum beta-lactamases. *Journal of Medical Microbiology*. **60** (1) 135-137.
- Solomon, E.B., B.A. Niemira, G.M. Sapers and B.A. Annous, (2005) Biofilm formation, cellulose production, and curli biosynthesis by *Salmonella* originating from produce, animal, and clinical sources. *Journal of Food Protection*. **68** (5) 906-912.
- Song, W., H. Lee, K. Lee, S.H. Jeong, I.K. Bae, J.S. Kim and H.S. Kwak, (2009) CTX-M-14 and CTX-M-15 enzymes are the dominant type of extended-spectrum beta-lactamase in clinical isolates of *Escherichia coli* from Korea. *Journal of Medical Microbiology*. **58** (Pt 2) 261-266.
- Sorensen, S.J., M. Bailey, L.H. Hansen, N. Kroer and S. Wuertz, (2005) Studying plasmid horizontal transfer *in-situ*: a critical review. *Nature Reviews Microbiology*. **3** (9) 700-710.
- Sorum, H. and M. Sunde, (2001). Resistance to antibiotics in the normal flora of animals. *Veterinary Research*. **32** (3-4) 227-241.

- Speldooren, V., B. Heym, R. Labia and M.H. Nicolas-Chanoine, (1998) Discriminatory detection of inhibitor-resistant beta-lactamases in *Escherichia coli* by single-strand conformation polymorphism-PCR. *Antimicrobial Agents and Chemotherapy*. **42** (4) 879-884.
- Srimanote, P., A.W. Paton and J.C. Paton, (2002) Characterization of a novel type IV pilus locus encoded on the large plasmid of locus of enterocyte effacement-negative Shiga-toxigenic *Escherichia coli* strains that are virulent for humans. *Infection and Immunity*. **70** (6) 3094-3100.
- Stewart, F.M. and B.R. Levin, (1977) The population biology of bacterial plasmids: A priori conditions for the existence of conjugationally transmitted factors. *Genetics*. **87** (2) 209-228.
- Stockwell, V.O. and J.E. Loper, (2005) The sigma factor RpoS is required for stress tolerance and environmental fitness of *Pseudomonas fluorescens* Pf-5. *Microbiology*. **151** (9) 3001-3009.
- Stokes, M., J.L. Cottell, L.J.V. Piddock, G. Wu, M. Wootton, L. Randall, C. Teale, M. Fielder and N.G. Coldham. Dissemination of *bla*_{CTX-M-14} by the IncK pCT plasmid vector in *E. coli* isolates from humans, turkeys and cattle in the UK. Submitted to *Emerging Infectious diseases*.
- Subbiah, M., E.M. Top, D.H. Shah and D.R. Call, (2011) The long-term persistence of *bla*_{CMY-2} positive, IncA/C plasmids requires selection pressure. *Applied Environmental Microbiology*. **77** (13) 4486-4493.
- Summers, D., (1998) Timing, self-control and a sense of direction are the secrets of multicopy plasmid stability. *Molecular Microbiology*. **29** (5) 1137-1145.
- Suzuki, H., H. Yano, C.J. Brown and E.M. Top, (2010) Predicting Plasmid Promiscuity Based on Genomic Signature. *Journal of Bacteriology*. **192** (22) 6045-6055.
- Suzuki, S., N. Shibata, K. Yamane, J. Wachino, K. Ito and Y. Arakawa, (2009) Change in the prevalence of extended-spectrum-beta-lactamase-producing *Escherichia coli* in Japan by clonal spread. *Journal of Antimicrobial Chemotherapy*. **63** (1) 72-79.
- Talbot, G.H., (2008) What is in the pipeline for Gram-negative pathogens? *Expert Review of Anti-Infective Therapy*. **6** (1) 39-49.
- Tamura, K., J. Dudley, M. Nei and S. Kumar, (2007) MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Molecular Biology and Evolution*. **24** (8) 1596-1599.
- Tangden, T., O. Cars, A. Melhus and E. Lowdin, (2010) Foreign Travel Is a Major Risk Factor for Colonization with *Escherichia coli* Producing CTX-M-Type Extended-Spectrum beta-lactamases: a Prospective Study with Swedish Volunteers. *Antimicrobial Agents and Chemotherapy*. **54** (9) 3564-3568.
- Teale, C.J., L. Barker, A. Foster, E. Liebana, M. Batchelor, D.M. Livermore and E.J. Threlfall, (2005) Extended-spectrum beta-lactamase detected in *E.coli* recovered from calves in Wales. *Veterinary records*. **156**, 186-187.

- Tenor, J.L., B.A. McCormick, F.M. Ausubel and A. Aballay, (2004) *Caenorhabditis elegans*-based screen identifies *Salmonella* virulence factors required for conserved host-pathogen interactions. *Current Biology*. **14** (11) 1018-1024.
- Thomas, J.R., J.C.B. DeNap, M.L. Wong and P.J. Hergenrother, (2005) The Relationship between Aminoglycosides' RNA Binding Proclivity and Their Antiplasmid Effect on an IncB Plasmid. *Biochemistry*. **44** (18) 6800-6808.
- Tian, S.F., B.Y. Chen, Y.Z. Chu and S. Wang, (2008) Prevalence of rectal carriage of extended-spectrum beta-lactamase-producing *Escherichia coli* among elderly people in community settings in China. *Canadian Journal of Microbiology*. **54** (9) 781-785.
- Timoney, J.F. and A.H. Linton, (1982) Experimental ecological studies on H2 plasmids in the intestine and faeces of the calf. *Journal of Applied Bacteriology*. **52**, 417-424.
- Tipper, D.J., (1985) Mode of action of beta-lactam antibiotics. *Pharmacology & Therapeutics*. **27** (1) 1-35.
- Trzcinski, K., C.M. Thompson, A.M. Gilbey, C.G. Dowson and M. Lipsitch, (2006) Incremental Increase in Fitness Cost with Increased beta-Lactam Resistance in *Pneumococci* Evaluated by Competition in an Infant Rat Nasal Colonization Model. *Journal of Infectious Diseases*. **193** (9) 1296-1303.
- Tschape, H. and E. Tietze, (1980) Genetic and molecular characterization of R plasmids incompatible with R387 (IncK). *Journal of General Microbiology*. **118** (2) 515-521.
- Turner, P.E., V.S. Cooper and R.E. Lenski, (1998) Tradeoff between horizontal and vertical modes of transmission in bacterial plasmids. *Evolution*. **52** (2) 315 - 329.
- Valverde, A., R. Canton, M.P. Garcillan-Barcia, A. Novais, J.C. Galan, A. Alvarado, F. de la Cruz, F. Baquero and T.M. Coque, (2009) Spread of *bla*_{CTX-M-14} is driven mainly by IncK plasmids disseminated among *Escherichia coli* phylogroups A, B1, and D in Spain. *Antimicrobial Agents and Chemotherapy*. **53** (12) 5204-5212.
- Valverde, A., T.M. Coque, M.P. Sanchez-Moreno, A. Rollan, F. Baquero and R. Canton, (2004) Dramatic increase in prevalence of faecal carriage of extended-spectrum beta-lactamase-producing *Enterobacteriaceae* during nonoutbreak situations in Spain. *Journal of Clinical Microbiology*. **42** (10) 4769-4775.
- Villa, L., A. Garcia-Fernandez, D. Fortini and A. Carattoli, (2010) Replicon sequence typing of IncF plasmids carrying virulence and resistance determinants. *Journal of Antimicrobial Chemotherapy*. **65** (12) 2518-2529.
- Vinue, L., M. Lantero, Y. Saenz, S. Somalo, I. de Diego, F. Perez, F. Ruiz-Larrea, M. Zarazaga and C. Torres, (2008) Characterization of extended-spectrum beta-lactamases and integrons in *Escherichia coli* isolates in a Spanish hospital. *Journal of Medical Microbiology*. **57** (Pt 7) 916-920.
- von Seidlein, L., D.R. Kim, M. Ali, H. Lee, X. Wang, V.D. Thiem, D.G. Canh, W. Chaicumpa, M.D. Agtini, A. Hossain, Z.A. Bhutta, C. Mason, O. Sethabutr, K. Talukder, G.B. Nair, J.L. Deen, K. Kotloff and J. Clemens, (2006) A Multicentre Study of *Shigella* Diarrhoea in Six Asian Countries: Disease Burden, Clinical Manifestations, and Microbiology. *PLoS Medicine*. **3** (9) e353.

- Wado, C., C. Imai and T. Yura, (1987) Host control of plasmid replication: Requirement for the δ factor $\delta 32$ in transcription of mini-F replication initiator gene. *Proceedings of the National Academy of Sciences of the USA*. **84**, 8849-8853.
- Walsh, T.R., (2008) Clinically significant carbapenemases: an update. *Current Opinion in Infectious Diseases*. **21** (4) 367.
- Walsh, T.R., J. Weeks, D.M. Livermore and M.A. Toleman, (2011) Dissemination of NDM-1 positive bacteria in the New Delhi environment and its implications for human health: an environmental point prevalence study. *The Lancet Infectious Diseases*. **11** (5) 355-362.
- Wang, Z., M. Gerstein and M. Snyder, (2009) RNA-Seq: a revolutionary tool for transcriptomics. *Nature Reviews Genetetics*. **10** (1) 57-63.
- Wardal, E., E. Sadowy and W. Hryniewicz, (2010) Complex nature of *Enterococcal* pheromone-responsive plasmids. *Polish Journal of Microbiology*. **59** (2) 79-87.
- Warren, R.E., V.M. Ensor, P. O'Neill, V. Butler, J. Taylor, K. Nye, M. Harvey, D.M. Livermore, N. Woodford and P.M. Hawkey, (2008) Imported chicken meat as a potential source of quinolone-resistant *Escherichia coli* producing extended-spectrum beta-lactamases in the UK. *Journal of Antimicrobial Chemotherapy*. **61** (3) 504-508.
- Watanabe, T., (1963) Infective heredity of multiple drug resistance in bacteria. *Bacteriology Reviews*. **27**, 87-115.
- Wendland, M. and D. Bumann, (2002) Optimization of GFP levels for analyzing *Salmonella* gene expression during an infection. *FEBS Letters*. **521** (1-3) 105-108.
- Williams, J.J. and P.J. Hergenrother, (2008) Exposing plasmids as the Achilles' heel of drug-resistant bacteria. *Current Opinion in Chemical Biology*. **12** (4) 389 - 399.
- Wong Ng, J., D. Chatenay, J. Robert and M.G. Poirier, (2010) Plasmid copy number noise in monoclonal populations of bacteria. *Physical Review*. **81** (1) 011909.
- Woodford, N., A. Carattoli, E. Karisik, A. Underwood, M.J. Ellington and D.M. Livermore, (2009) Complete nucleotide sequences of plasmids pEK204, pEK499 and pEK516, encoding CTX-M enzymes in three major *Escherichia coli* lineages from the United Kingdom, all belonging to the international O25:H4-ST131 clone. *Antimicrobial Agents and Chemotherapy*. **53** (10) 4472-4482.
- Woodford, N., M.E. Ward, M.E. Kaufmann, J. Turton, E.J. Fagan, D. James, A.P. Johnson, R. Pike, M. Warner, T. Cheasty, A. Pearson, S. Harry, J.B. Leach, A. Loughrey, J.A. Lowes, R.E. Warren and D.M. Livermore, (2004) Community and hospital spread of *Escherichia coli* producing CTX-M extended-spectrum beta-lactamases in the UK. *Journal of Antimicrobial Chemotherapy*. **54** (4) 735-743.
- Wray, C. and W.J. Sojka, (1978) Experimental *Salmonella* Typhimurium infection in calves. *Research in Veterinary Science*. **25** (2) 139-143.
- Xu, L., S. Shabir, T. Bodah, C. McMurray, K. Hardy, P. Hawkey and K. Nye, (2011) Regional survey of CTX-M-type extended-spectrum beta-lactamases among *Enterobacteriaceae* reveals marked heterogeneity in the distribution of the ST131 clone. *Journal of Antimicrobial Chemotherapy*. **66** (3) 505-511.

- Yates, C.M., D.J. Shaw, A.J. Roe, M.E.J. Woolhouse and S.G.B. Amyes, (2006) Enhancement of bacterial competitive fitness by apramycin resistance plasmids from non-pathogenic *Escherichia coli*. *Biology Letters*. **2** (3) 463 - 465.
- Yi, H., Y. Xi, J. Liu, J. Wang, J. Wu, T. Xu, W. Chen, B. Chen, M. Lin, H. Wang, M. Zhou, J. Li, Z. Xu, S. Jin and Q. Bao, (2010) Sequence analysis of pKF3-70 in *Klebsiella pneumoniae*: probable origin from R100-like plasmid of *Escherichia coli*. *PLoS One*. **5** (1) e8601.
- Yoshida, T., S.R. Kim and T. Komano, (1999) Twelve pil genes are required for biogenesis of the R64 thin pilus. *Journal of Bacteriology*. **181** (7) 2038-2043.
- Yu, W.L., P.L. Winokur, D.L. Von Stein, M.A. Pfaller, J.H. Wang and R.N. Jones, (2002) First description of *Klebsiella pneumoniae* harboring CTX-M beta-lactamases (CTX-M-14 and CTX-M-3) in Taiwan. *Antimicrobial Agents and Chemotherapy*. **46** (4) 1098-1100.
- Zaslaver, A., A. Bren, M. Ronen, S. Itzkovitz, I. Kikoin, S. Shavit, W. Liebermeister, M.G. Surette and U. Alon, (2006). A comprehensive library of fluorescent transcriptional reporters for *Escherichia coli*. *Nature Methods*. **3** (8) 623-628.
- Zong, Z., S.R. Partridge, L. Thomas and J.R. Iredell, (2008) Dominance of *bla*_{CTX-M} within an Australian extended-spectrum beta-lactamase gene pool. *Antimicrobial Agents and Chemotherapy*. **52** (11) 4198-4202.
- Zund, P. and G. Lebek, (1980) Generation time-prolonging R plasmids: correlation between increases in the generation time of *Escherichia coli* caused by R plasmids and their molecular size. *Plasmid*. **3** (1) 65-69.